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(54) Title: USE OF GENETIC POLYMORPHISMS THAT ASSOCIATE WITH EFFICACY OF TREATMENT OF INFLAMMATORY DISEASE

(57) Abstract: The MHC III region of chromosome 6p21.3 harbors a DNA sequence, most likely within the TNF or LTA gene, which influences response to pimecrolimus for the treatment of atopic dermatitis. Accordingly, genetic polymorphisms in the TNF and LTA genes are useful as biomarkers of the efficacy of pimecrolimus treatment of inflammatory disease. By contrast, response to tacrolimus appears to be influenced by other biological pathways.

USE OF GENETIC POLYMORPHISMS THAT ASSOCIATE WITH EFFICACY OF TREATMENT OF INFLAMMATORY DISEASE

FIELD OF THE INVENTION

[0001]   This invention relates generally to the analytical testing of tissue samples *in vitro*, and more particularly to the analysis of genetic polymorphisms as biomarkers for the efficacy of treatments of inflammatory disease.

DESCRIPTION OF THE RELATED ART

[0002]   Atopic dermatitis (AD), also known as eczema, is an inflammatory skin disease typified by itchy inflamed skin that presents during infancy and childhood. The disease is one of the most common dermatological disorders in Western countries, affecting as many as 20% of children under the age of five. Cookson WO *et al., Nature Genetics* 27: 372-373 (2001).

[0003]   Pimecrolimus (Elidel®) is an ascomycin macrolactam derivative that was specifically developed for the treatment of inflammatory skin diseases. Tacrolimus (FK506; Protopic® or Prograf®) is a member of the same class of compounds and is also used to treat inflammatory skin diseases. Both pimecrolimus and tacrolimus interfere with the inflammatory process by binding with high affinity to macrophilin-12 and inhibiting calcineurin, which blocks transcription of Th1- and Th2-type cytokines in T lymphocytes. While the two compounds are structurally similar and have comparable mechanisms of action, pimecrolimus has a higher affinity for skin than does tacrolimus.

[0004]   It has long been recognized that there is a genetic basis to atopic diseases. Several linkage analyses have been done to define susceptibility regions that contribute to atopic dermatitis, as reviewed in Maclean JA & Eidelman FJ, *Arch Dermatol* 137: 1474-1476 (2001). At least five chromosomal regions (3q21, 5q31-33, 11q13, 13q12-14 and 14q11.2) are thought to contain atopic dermatitis susceptibility loci. Current thinking is that atopic mechanisms are primarily responsible for the pathogenesis of the atopic dermatitis. However, other genes involved with dermal inflammation may also contribute to the condition. Cookson WO *et al., Nature Genetics* 27: 372-373 (2001).

[0005]   The response rate among patients taking topical macrolactams (pimecrolimus or tacrolimus) is currently less than 50%. It is not known why some patients do not respond to these medications. Thus, there remains a need in the art for the improving the efficacy of

topical macrolactams, such as by targeting appropriate formulations of the medications to those inflammatory disease patients who are genetically disposed to respond and benefit from the treatment.

SUMMARY OF THE INVENTION

[0006]   The invention provides a method for treating atopic dermatitis, based upon a determination of the patient's *TNF* alleles. In one embodiment, patients are tested for the presence of a (-1031) *TNF* polymorphism to determine what therapy is appropriate. Patients having the TT alleles (SEQ ID NO:1) at that locus are treated with pimecrolimus cream or ointment, while patients having the CC alleles (SEQ ID NO:2) or the CT alleles (SEQ ID NOS:1 and 2) are treated with a higher dose of pimecrolimus cream, with pimecrolimus cream plus another anti-inflammatory drug, or with an anti-inflammatory drug without pimecrolimus cream.

[0007]   The invention also provides a general method for treating psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or any disorder for which pimecrolimus (oral, topical or other) is indicated, based upon a determination of the patient's *TNF* alleles. In one embodiment, patients are tested for the presence of a (-1031) *TNF* polymorphism to determine what therapy is appropriate. Patients with TT alleles at that locus are treated with pimecrolimus, while patients with CC alleles and CT alleles are given a higher dose of pimecrolimus, pimecrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without pimecrolimus.

[0008]   The invention further provides a method for treating psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or any disorder for which pimecrolimus (oral, topical or other) is indicated, based upon a determination of the patient's *LTA* alleles. In one embodiment, patients are tested for the presence of an ASN60THR *LTA* locus polymorphism to determine what therapy is appropriate. Patients with AA alleles (SEQ ID NO:3) or AC alleles (SEQ ID NOS:3 and 4) at that locus are treated with pimecrolimus, while patients with CC alleles (SEQ ID NO:4) are given a higher dose of pimecrolimus, pimecrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without pimecrolimus.

[0009]   The invention also provides a method for treating psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or any disorder for which tacrolimus (oral, topical or other) is indicated, based upon a determination of the patient's *CCR2*

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alleles. In one embodiment, patients are tested for the presence of a VAL64ILE *CCR2* locus polymorphism to determine what therapy is appropriate. Patients with GG alleles (SEQ ID NO:5) at that locus are treated with tacrolimus, while patients with AG alleles (SEQ ID NOS:5 and 6) are given a higher dose of tacrolimus, tacrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without tacrolimus.

[0010]   The invention provides a method for treating atopic dermatitis or any disorder for which pimecrolimus (oral, topical or other) is indicated, where the level of TNF-α protein or mRNA present in the patient is measured prior to determining what therapy is appropriate. In one embodiment, when TNF-α protein or mRNA levels in the bodily fluids or non-inflamed tissue samples of the patient are low or normal, then the patient is treated with pimecrolimus. In another embodiment, when TNF-α protein or mRNA levels are elevated, then the patient is treated with pimecrolimus in combination with another anti-inflammatory drug or with an alternative therapy.

[0011]   The invention further provides a method for determining a treatment strategy for a condition in a subject, wherein the condition is selected from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for which pimecrolimus is indicated, said method comprises analyzing the level of TNF-α mRNA or TNF-α protein in a sample obtained from the subject,; and wherein the treatment strategy comprises the selection of a pimecrolimus formulation as treatment when the level of TNF-α mRNA or TNF-α protein in the sample is low or normal; or the selection of either (A) a pimecrolimus formulation in combination with another immunosuppressant or (B) another immunosuppressant as treatment when the level of TNF-α mRNA or TNF-α protein in the sample is elevated. Preferably, said sample is taken from non-inflamed tissue.

[0012]   The invention provides a method for choosing subjects for inclusion in a clinical trial of inflammatory skin disease or any other disorder for which pimecrolimus is indicated, where genetic polymorphisms at the (-1031) TNF locus, the ASN60THR *LTA* locus or the VAL64ILE *CCR2* locus of the candidate subjects are interrogated prior to inclusion in the clinical trial.

[0013]   The invention provides a kit for use in determining treatment strategy for a patient with inflammatory skin disease or any other disorder for which pimecrolimus is indicated. In one embodiment, the kit contains reagents for determining the levels of TNF-

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α protein or mRNA in a sample obtained from a subject to be treated. In another embodiment, the kit contains reagents for determining the genotype of the subject in the *TNF*, *LTA* or *CCR2* genes. The kit also contains a written product describing the use of the biomarker in determining a treatment strategy for the condition.

[0014]   The invention further provides a method for determining a treatment strategy for a condition in a subject, wherein the condition is selected from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for which pimecrolimus or tacrolimus is indicated, said method comprises analyzing the genotype of a subject at a genetic locus from the TNF gene cluster indicative of efficacy of a selected macrolactam formulation in treating the condition. Preferably, the genotype is determined by analyzing a sample obtained from said subject. In one embodiment, subjects / patients are tested for the presence of a (-1031) *TNF* polymorphism to determine the appropriate treatment strategy. For patients with TT alleles (SEQ ID NO:1) at that locus pimecrolimus is selected as treatment, while for patients with CC alleles (SEQ ID NO:2) and CT alleles (SEQ ID NOS:1 and 2) a higher dose of pimecrolimus, pimecrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without pimecrolimus is selected as treatment. Another embodiment of the invention provides that said treatment strategy is based upon a determination of the patient's *LTA* alleles. In a preferred embodiment, patients are tested for the presence of an ASN60THR *LTA* locus polymorphism to determine the treatment strategy. For patients with AA alleles (SEQ ID NO:3) or AC alleles (SEQ ID NOS:3 and 4) at that locus pimecrolimus is selected as treatment, while for patients with CC alleles (SEQ ID NO:4) a higher dose of pimecrolimus, pimecrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without pimecrolimus is selected as treatment. The anti-inflammatory drug is preferably selected from the group consisting of hydrocortisone, cyclosporine, tacrolimus and sirolimus.

[0015]   The invention provides a further method for determining a treatment strategy for a subject / patient affected by atopic dermatitis, psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for which pimecrolimus or tacrolimus is indicated, based upon a determination of the patient's *CCR2* alleles. In one embodiment, the patient is tested for the presence of a VAL64ILE *CCR2* locus polymorphism to determine the appropriate treatment strategy. For a patient with GG allele (SEQ ID NO:5) at that locus tacrolimus is selected as treatment strategy, while for a patient with AG allele

(SEQ ID NOS:5 and 6) a higher dose of tacrolimus, tacrolimus plus another anti-inflammatory drug or an anti-inflammatory drug without tacrolimus is selected as treatment strategy. . The anti-inflammatory drug is preferably selected from the group consisting of hydrocortisone, cyclosporine, pimecrolimus and sirolimus.

[0016]   The invention also provides the use of pimecrolimus in the manufacture of a medicament for the treatment of inflammatory disease in a selected patient population, wherein the patient population is selected on the basis of the genotype of the patients at a genetic locus from the TNF gene cluster indicative of efficacy of pimecrolimus in treating inflammatory disease. In one embodiment, the genotype of the selected patient population comprises the presence of a (-1031) *TNF* polymorphism. Preferably, the selected patient population comprises TT alleles at that locus. In another embodiment, the selected patient population comprises an ASN60THR *LTA* locus polymorphism. Preferably, the selected patient population comprises AA alleles or AC alleles at that locus.

[0017]   Further, the invention provides the use of tacrolimus in the manufacture of a medicament for the treatment of inflammatory disease in a selected patient population, wherein the patient population is selected on the basis of the genotype of the patients at the *CCR2* genetic locus indicative of efficacy of tacrolimus in treating inflammatory disease. In one embodiment, the selected patient population comprises the VAL64ILE *CCR2* locus polymorphism. Preferably, said patient population comprise GG alleles at that locus.

[0018]   The invention thus provides a way of improving the efficacy of topical macrolactams, particularly pimecrolimus, by identifying biomarkers of efficacy in the subjects to be treated and then targeting appropriate macrolactam formulations to those who are best able to respond and benefit from the treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]   FIG. 1 is a diagrammatic representation of the major histocompatability complex (MHC) on chromosome (Ch) 6 showing the placement of LTA (gene coding for lymphotoxin alpha) and TNF (gene coding for tumor necrosis factor alpha; TNF-α) within the MHC. Shown in greater detail below is the region of 6p21.3 that contains the TNF gene cluster. * denotes polymorphisms, which are roughly 2.5 kb apart, discussed herein. This figure is modified from Field M, *Q J Med* 94: 237-246 (2001).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0020]   The efficacy of treatment of atopic dermatitis by pimecrolimus cream 1%, but
not treatment by tacrolimus ointment 0.03%, is associated with polymorphic markers in the
TNF cluster on 6p21.3. This discovery provides a way to use genetic polymorphisms that
associate with the efficacy of pimecrolimus treatment in determining the most effective
treatment.

[0021]   The genetic locus was identified in a Phase IIIb trial that was designed as a head
to head comparison of pimecrolimus cream 1% and tacrolimus ointment 0.03%. The
six-week investigator-blinded trial of pediatric subjects with moderate atopic dermatitis
explored the incidence of local application site reactions induced by the two topical
macrolactams, as well as their efficacy, safety and cosmetic acceptability. Blood samples
for pharmacogenetic analysis were collected from consenting participants in the clinical
trial, to define a genetic basis for response to either treatment.

[0022]   Among those genes selected for pharmacogenomic analysis were *LTA*, which
codes for lymphotoxin alpha (LTα), and *TNF*, which codes for tumor necrosis factor alpha
(TNF-α). The *LTA* and *TNF* genes are tandemly located in the gene-rich MHCIII region of
6p21.3 (see, FIG. 1). Both genes contain multiple polymorphisms, some of which have
been well-characterized, as reviewed by Field M, *Q J Med* 94: 237-246 (2001). Both the
LTA and the *TNF* gene products are cytokines involved in inflammatory processes. Both
cytokines signal through TNF-α receptors, which are expressed in many different tissues,
including the skin. Rulls SR & Sedgwick JD, *Am J Hum Genet* 65: 294-301 (1999).
Furthermore, both *LTA* and *TNF* have been implicated in the pathogenesis of atopic disease.
Polymorphisms in both these genes are associated with atopy, particularly asthma. Trabetti
E *et al., J Med Genet* 36: 323-5 (1999); Izakovicaova Holla L *et al., Clin Exp Allergy* 9:
1418-23 (2001); Castro J *et al., J Investig Allergol Clin Immunol* 3:149-5 (2000); Li Kam
Wa TC *et al., Clin Exp Allergy* 29: 1204-8 (1999); and Zhu S *et al. Am J Respir Crit Care
Med* 161: 1655 – 9 (2000). TNF-α has been extensively studied for its role in dermal
inflammation. Both dermal mast cells and keratinocytes produce TNF-α. While expression
of TNF-α is constitutive in the skin, the level of expression can be further induced by a
variety of stimuli. TNF-α levels are elevated in skin lesions of psoriasis, and several reports
have suggested that drugs that modulate TNF-α function are effective in the treatment of
psoriasis, as reviewed by LaDuca JR & Gaspari AA, *Dermatol Clin* 19: 617-635 (2001) and

Mease PJ, *Ann Rheum Dis* 61: 298-304 (2002). Though psoriasis is distinct from atopic dermatitis, the expression of both diseases is influenced by genes that modulate dermal inflammation. Cookson WO *et al.*, *Nature Genetics* 27: 372-373 (2001).

[0023]   In the clinical trial and subsequent pharmacogenomics analysis, an association between pimecrolimus efficacy and the (-1031) *TNF* polymorphism was found in pediatric subjects (ages 2-17 years) with atopic dermatitis who were treated topically with the cream formulation of pimecrolimus. The polymorphism that associated with pimecrolimus efficacy is located –1031 from the transcription start site of the gene and changes the wild-type T to a C. The only subjects that experienced efficacy in this trial had a TT genotype at (-1031) *TNF* (SEQ ID NO:1). No patient with a C allele (CC (SEQ ID NO:2) or CT(SEQ ID NOS:1 and 2)) at that locus responded to pimecrolimus. This association did not hold for tacrolimus.

[0024]   For a second SNP in the same chromosomal region, ASN60THR *LTA*, the data also suggested a trend toward significance.

[0025]   These associations can reasonably be expected to hold for all age groups and for subjects with other inflammatory skin diseases. Moreover, the efficacy of pimecrolimus can reasonably be predicted when the compound is formulated as a tablet or aerosol. The method can be used for vertebrate subjects, particularly mammalian subjects and more particularly for human subjects.

[0026]   Individuals carrying polymorphic alleles of interest (*i.e.*, in genes from the *TNF* cluster, such as *TNF* and *LTA*) may be detected at the DNA, the RNA, or the protein level using a variety of techniques that are well known in the art. Strategies for identification and detection are described in *e.g.* EP 730,663, EP 717,113, and PCT US97/02102. The methods of the invention may involve the detection of pre-characterized polymorphisms, where the genotyping location and nature of polymorphic forms present at a site have already been determined (see, discussion above regarding *LTA* and *TNF* genes). The availability of this information allows sets of probes to be designed for specific identification of the known polymorphic forms. The identification of alleles containing single nucleotide polymorphisms may involve the amplification of DNA from target samples. This can be accomplished by *e.g.*, PCR. See generally *PCR Technology: Principles and Applications for DNA* Amplification, (ed. Erlich, Freeman Press, NY, N.Y., 1992); *PCR Protocols: A Guide to Methods and Applications* (eds. Innis, *et al.*, Academic Press, San Diego, Calif., 1990). The detection of polymorphisms in specific DNA

sequences, can be accomplished by a variety of methods including, but not limited to, restriction-fragment-length-polymorphism detection based on allele-specific restriction-endonuclease cleavage (Kan & Dozy, *Lancet* II:910-912 (1978)), hybridization with allele-specific oligonucleotide probes (Wallace *et al, Nucl. Acids Res.* 6:3543-3557 (1978)), including immobilized oligonucleotides (Saiki *et al., Proc. Natl. Acad. Sci. USA*, 86:6230-6234 (1969)) or oligonucleotide arrays (Maskos & Southern, *Nucl. Acids Res.* 21:2269-2270 (1993)), allele-specific PCR (Newton *et al., Nucl. Acids Res.* 17:2503-2516 (1989)), mismatch-repair detection (MRD) (Faham & Cox, *Genome Res.* 5:474-482 (1995)), binding of MutS protein (Wagner *et al., Nucl. Acids Res.* 23:3944-3948 (1995), denaturing-gradient gel electrophoresis (DGGE) (Fisher & Lerman, *Proc. Natl. Acad. Sci. U.S.A.* 80:1579-1583 (1983)), single-strand-conformation-- polymorphism detection (Orita *et al., Genomics* 5:874-879 (1983)), RNAse cleavage at mismatched base-pairs (Myers *et al., Science* 230:1242 (1985)), chemical (Cotton *et al., Proc. Natl. Acad. Sci. U.S.A.*, 8Z:4397-4401 (1988)) or enzymatic (Youil *et al., Proc. Natl. Acad. Sci. U.S.A.* 92:87-91 (1995)) cleavage of heteroduplex DNA, methods based on allele specific primer extension (Syvanen *et al., Genomics* 8:684-692 (1990)), genetic bit analysis (GBA) (Nikiforov *et al., Nucl. Acids Res.* 22:4167-4175 (1994)), the oligonucleotide-ligation assay (OLA) (Landegren *et al., Science* 241:1077 (1988)), the allele-specific ligation chain reaction (LCR) (Barrany, *Proc. Natl. Acad. Sci. U.S.A.* 88:189-193 (1991)), gap-LCR (Abravaya *et al., Nucl. Acids Res.* 23:675-682 (1995)), radioactive and/or fluorescent DNA sequencing using standard procedures well known in the art, and peptide nucleic acid (PNA) assays (Orum *et al., Nucl. Acids Res.* 21:5332-5356 (1993); Thiede *et al., Nucl. Acids Res.* 24:983-984 (1996)). Additional guidance is provided by Sambrook J *et al., Molecular Cloning: A Laboratory Manual, Third Edition* (Cold Spring Harbor Press, Cold Spring Harbor, 2000).

[0027]   Levels of TNF-α mRNA or protein can be determined by methods known to those of skill in the art. See, U.S. Pat. Nos. 6,566,501; 6,541,620; and 6,537,540. A discussion of the levels of the level of tumor necrosis factor in TNF-associated disorders is provided in U.S. Pat. No. 6,537,540 and references cited therein (all of which are incorporated by reference.).

[0028]   As used herein, the level of TNF-α mRNA (SEQ ID NO:7) in the sample is determined to be "high" when the ratio of TNF-α mRNA to β-actin expression (SEQ ID NO:9; see, Actor JK *et al., Comb Chem High Throughput Screen* 3(4):343-51 (August 2000)) or GAPDH expression (see, Anderson GD *et al., J Clin Invest.* 97(11):2672-9 (June

1, 1996)) in the sample is about twice as high or higher than the ratio of TNF-α mRNA to β-actin expression, as compared with a sample from an individual (or preferably a population of individuals) who does not have an inflammatory skin condition. As used herein, the level of TNF-a mRNA in the sample is determined to be "low or normal" when the ratio of TNF-α mRNA to β-actin expression in the sample is about equal to or less than the ratio of TNF-α mRNA to β-actin expression, as compared with a sample from an individual (or preferably, a population of individuals) who does not have an inflammatory skin condition. For guidance as to which TNF-α mRNA and protein levels tissue are generally expected to be "low", "normal" or "high", see, Van Deventer SJH, *Gut* 40: 443-8 (1997); McAlindon ME & Mahida YR, *Aliment Pharmacol Ther* 10(suppl 2): 72-4 (1996); Reimund J-M *et al.*, *J Clin Immunol* 16: 144-50 (1996); Reinecker H-C *et al.*, *Clin Exp Immunol* 94: 174-81 (1993); Murch SH *et al.*, *Gut.* 34: 1705-9 (1993) and Grom AA *et al.*, *Arthritis Rheum* 39: 1703-1710 (1996). Alternatively, one of skill in the art can define an average or expected level of TNF-α mRNA expression in a population, *e.g.*, by performing the assay in ten or more control subjects (such as by methods shown in the EXAMPLE) and obtaining the average and standard deviation.

[0029]   Samples for the determination of genetic polymorphisms, *TNF* mRNA levels (SEQ ID NO:7) or TNF protein levels (SEQ ID NO:8) can be collected from subjects by any appropriate method know to those of skill in the medical art. Samples can be fluid samples, such as from blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen or other bodily fluid. Alternatively, samples can be tissue samples, such as from biopsy samples, homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof. A sample can also be a medium, such as a nutrient broth or gel in which an organism has been propagated, which contains cellular components, such as proteins or nucleic acid molecules.

[0030]   Guidance for the use of pimecrolimus cream 1% is provided by Novartis Pharmaceuticals Corp., Elidel® Prescribing Information, T2003-01, 89014902, 492573/1 US (Rev. April 2003). Additional guidance for the use of pimecrolimus is provided by Nghiem P *et al.*, *J Am Acad Dermatol* 46: 228-241 (2002) and references cited therein (all of which are incorporated by reference) and in U.S. Pat. Nos. 5,912,238; 6,352,998 and 6,423,722.

[0031]   Guidance for the use of tacrolimus ointment (0.03% or 0.1%) is provided by Fujisawa Healthcare Inc., Protopic® Prescribing Information, 45670 (December 2000). Guidance for the use of Prograf® (oral capsule or injectable) is provided by Fujisawa Healthcare Inc., Prograf® Prescribing Information, ZL40306 and Fujisawa Healthcare Inc., Prograf® Product Monograph. Additional guidance for the use of tacrolimus is provided by Nghiem P *et al., J Am Acad Dermatol* 46: 228-241 (2002) and references cited therein (all of which are incorporated by reference).

[0032]   As used herein, a "high dose of pimecrolimus" is a dose substantially higher than the recommended dose of pimecrolimus. For example, for adults and children over two years of age, a thin layer of pimecrolimus cream 1% is applied to the affected area twice times a day, then rubbed in gently. A dose substantially higher than the recommended dose may be the application four times a day. Alternatively, a dose substantially higher than the recommended dose may be the application twice a day of a pimecrolimus formulation with a concentration of 2% or higher, if such be made available. Likewise, a "high dose of tacrolimus" is a dose substantially higher than the recommended dose of tacrolimus.

[0033]   The use of other immunosuppressants, such as hydrocortisone, cyclosporine or sirolimus (rapamycin), in dermatology generally and in particular for atopic dermatitis, is well-known to those of skill in the medical arts. See, Marsland AM & Griffiths CE, *Eur J Dermatol.* 2(6):618-22 (November-December 2002) and Nghiem P *et al., J Am Acad Dermatol* 46: 228-241 (2002).

[0034]   The diagnosis of atopic dermatitis is well-known to those of skill in the medical arts. See, Cookson WO *et al., Nature Genetics* 27: 372-373 (2001); Nghiem P *et al., J Am Acad Dermatol* 46: 228-241 (2002) and references cited therein (all of which are incorporated by reference).

[0035]   *Single Nucleotide Polymorphisms*. Sequence variation in the human genome consists primarily of single nucleotide polymorphisms ("SNPs") with the remainder of the sequence variations being short tandem repeats (including micro-satellites), long tandem repeats (mini-satellite) and other insertions and deletions. A SNP is a position at which two alternative bases occur at appreciable frequency (*i.e.* >1%) in the human population. A SNP is said to be "allelic" in that due to the existence of the polymorphism, some members of a species may have the unmutated sequence (i.e., the original "allele") whereas other members may have a mutated sequence (i.e., the variant or mutant allele). In the simplest case, only one mutated sequence may exist, and the polymorphism is said to be diallelic.

The occurrence of alternative mutations can give rise to triallelic polymorphisms, *etc.* SNPs are widespread throughout the genome and SNPs that alter the function of a gene may be direct contributors to phenotypic variation. Due to their prevalence and widespread nature, SNPs have potential to be important tools for locating genes that are involved in human disease conditions, *see e.g.*, Wang *et al., Science* 280: 1077-1082 (1998), which discloses a pilot study in which 2,227 SNPs were mapped over a 2.3 megabase region of DNA.

[0036]   An association between a single nucleotide polymorphisms and a particular phenotype does not indicate or require that the SNP is causative of the phenotype. Instead, such an association may indicate only that the SNP is located near the site on the genome where the determining factors for the phenotype exist and therefore is more likely to be found in association with these determining factors and thus with the phenotype of interest. Thus, a SNP may be in linkage disequilibrium (LD) with the 'true' functional variant. LD, also known as allelic association exists when alleles at two distinct locations of the genome are more highly associated than expected.

[0037]   Thus a SNP may serve as a marker that has value by virtue of its proximity to a mutation that causes a particular phenotype.

[0038]   SNPs that are associated with disease may also have a direct effect on the function of the gene in which they are located. A sequence variant may result in an amino acid change or may alter exon-intron splicing, thereby directly modifying the relevant protein, or it may exist in a regulatory region, altering the cycle of expression or the stability of the mRNA, see Nowotny P *Current Opinions in Neurobiology* 11:637-641 (2001).

[0039]   It is increasingly clear that the risk of developing many common disorders and the metabolism of medications used to treat these conditions are substantially influenced by underlying genomic variations, although the effects of any one variant might be small.

[0040]   Therefore, an association between a SNP and a clinical phenotype suggests, (1) the SNP is functionally responsible for the phenotype or, (2) there are other mutations near the location of the SNP on the genome that cause the phenotype. The 2nd possibility is based on the biology of inheritance. Large pieces of DNA are inherited and markers in close proximity to each other may not have been recombined in individuals that are unrelated for many generations, *i.e.*, the markers are in linkage disequlibrium (LD).

[0041]   *Identification and characterization of SNPs.* Many different techniques can be used to identify and characterize SNPs, including single-strand conformation

polymorphism analysis, heteroduplex analysis by denaturing high-performance liquid chromatography (DHPLC), direct DNA sequencing and computational methods, *see* Shi MM, *Clin Chem* 47:164-172 (2001). Thanks to the wealth of sequence information in public databases, computational tools can be used to identify SNPs *in silico* by aligning independently submitted sequences for a given gene (either cDNA or genomic sequences). Comparison of SNPs obtained experimentally and by *in silico* methods showed that 55% of candidate SNPs found by SNPFinder(http://Ipgws.nci.nih.gov:82/perl/snp/snp_cgi.pl) have also been discovered experimentally, *see*, Cox *et al. Hum Mutat* 17:141-150 (2001). However, these *in silico* methods could only find 27% of true SNPs.

[0042]   The most common SNP typing methods currently include hybridization, primer extension and cleavage methods. Each of these methods must be connected to an appropriate detection system. Detection technologies include fluorescent polarization, (see Chan X *et al. Genome Res* 9:492-499 (1999)), luminometric detection of pyrophosphate release (pyrosequencing), (see Ahmadiian A *et al., Anal Biochem* 280:103-10 (2000)), fluorescence resonance energy transfer (FRET)-based cleavage assays, DHPLC, and mass spectrometry, (see Shi MM, *Clin Chem* 47:164-172 (2001) and U.S. Pat. No. 6,300,076 B1). Other methods of detecting and characterizing SNPs are those disclosed in U.S. Patents No. 6,297,018 B1 and 6,300,063 B1. The disclosures of the above references are incorporated herein by reference in their entirety.

[0043]   In a particularly preferred embodiment the detection of the polymorphism can be accomplished by means of so called INVADER™ technology (available from Third Wave Technologies Inc. Madison, Wis.). In this assay, a specific upstream "invader" oligonucleotide and a partially overlapping downstream probe together form a specific structure when bound to complementary DNA template. This structure is recognized and cut at a specific site by the Cleavase enzyme, and this results in the release of the 5' flap of the probe oligonucleotide. This fragment then serves as the "invader" oligonucleotide with respect to synthetic secondary targets and secondary fluorescently labeled signal probes contained in the reaction mixture. This results in specific cleavage of the secondary signal probes by the Cleavase enzyme. Fluorescence signal is generated when this secondary probe , labeled with dye molecules capable of fluorescence resonance energy transfer, is cleaved. Cleavases have stringent requirements relative to the structure formed by the overlapping DNA sequences or flaps and can, therefore, be used to specifically detect single base pair mismatches immediately upstream of the cleavage site on the downstream

DNA strand. See Ryan D *et al. Molecular Diagnosis* 4(2):135-144 (1999) and Lyamichev V *et al. Nature Biotechnology* 17:292-296 (1999), see also US Patents 5,846,717 and 6,001,567 (the disclosures of which are incorporated herein by reference in their entirety).

[0044]   In some embodiments, a composition contains two or more differently labeled genotyping oligonucleotides for simultaneously probing the identity of nucleotides at two or more polymorphic sites. It is also contemplated that primer compositions may contain two or more sets of allele-specific primer pairs to allow simultaneous targeting and amplification of two or more regions containing a polymorphic site.

[0045]   Genotyping oligonucleotides of the invention may also be immobilized on or synthesized on a solid surface such as a microchip, bead. or glass slide (see, *e.g.,* WO 98/20020 and WO 98/20019). Such immobilized genotyping oligonucleotides may be used in a variety of polymorphism detection assays, including but not limited to probe hybridization and polymerase extension assays. Immobilized genotyping oligonucleotides of the invention may comprise an ordered array of oligonucleotides designed to rapidly screen a DNA sample for polymorphisms in multiple genes at the same time.

[0046]   An allele-specific oligonucleotide primer of the invention has a 3' terminal nucleotide, or preferably a 3' penultimate nucleotide, that is complementary to only one nucleotide of a particular SNP, thereby acting as a primer for polymerase-mediated extension only if the allele containing that nucleotide is present. Allele-specific oligonucleotide primers hybridizing to either the coding or noncoding strand are contemplated by the invention. An ASO primer for detecting gene polymorphisms could be developed using techniques known to those of skill in the art.

[0047]   Other genotyping oligonucleotides of the invention hybridize to a target region located one to several nucleotides downstream of one of the novel polymorphic sites identified herein. Such oligonucleotides are useful in polymerase-mediated primer extension methods for detecting one of the novel polymorphisms described herein and therefore such genotyping oligonucleotides are referred to herein as "primer-extension oligonucleotides". In a preferred embodiment, the 3'-terminus of a primer-extension oligonucleotide is a deoxynucleotide complementary to the nucleotide located immediately adjacent to the polymorphic site.

[0048]   In another embodiment, the invention provides a kit comprising at least two genotyping oligonucleotides packaged in separate containers. The kit may also contain other components such as hybridization buffer (where the oligonucleotides are to be used as

a probe) packaged in a separate container. Alternatively, where the oligonucleotides are to be used to amplify a target region, the kit may contain, packaged in separate containers, a polymerase and a reaction buffer optimized for primer extension mediated by the polymerase, such as PCR.

[0049]   The above described oligonucleotide compositions and kits are useful in methods for genotyping and/or haplotyping the gene in an individual. As used herein, the terms " genotype" and " haplotype" mean the genotype or haplotype containing the nucleotide pair or nucleotide, respectively, that is present at one or more of the novel polymorphic sites described herein and may optionally also include the nucleotide pair or nucleotide present at one or more additional polymorphic sites in the gene. The additional polymorphic sites may be currently known polymorphic sites or sites that are subsequently discovered.

[0050]   One embodiment of the genotyping method involves isolating from the individual a nucleic acid mixture comprising the two copies of the gene, or a fragment thereof, that are present in the individual, and determining the identity of the nucleotide pair at one or more of the polymorphic sites in the two copies to assign a genotype to the individual. As will be readily understood by the skilled artisan, the two "copies" of a gene in an individual may be the same allele or may be different alleles. In a particularly preferred embodiment, the genotyping method comprises determining the identity of the nucleotide pair at each polymorphic site.

[0051]   Typically, the nucleic acid mixture is isolated from a biological sample taken from the individual, such as a blood sample or tissue sample. Suitable tissue samples include whole blood, semen, saliva, tears, urine, fecal material, sweat, buccal smears, skin and hair. The nucleic acid mixture may be comprised of genomic DNA, mRNA, or cDNA and, in the latter two cases, the biological sample must be obtained from an organ in which the gene is expressed. Furthermore it will be understood by the skilled artisan that mRNA or cDNA preparations would not be used to detect polymorphisms located in introns or in 5' and 3' nontranscribed regions. If a gene fragment is isolated, it must contain the polymorphic site(s) to be genotyped.

[0052]   One embodiment of the haplotyping method comprises isolating from the individual a nucleic acid molecule containing only one of the two copies of the gene, or a fragment thereof, that is present in the individual and determining in that copy the identity of the nucleotide at one or more of the polymorphic sites in that copy to assign a haplotype

to the individual. The nucleic acid may be isolated using any method capable of separating the two copies of the gene or fragment, including but not limited to, one of the methods described above for preparing isogenes, with targeted *in vivo* cloning being the preferred approach. As will be readily appreciated by those skilled in the art, any individual clone will only provide haplotype information on one of the two gene copies present in an individual. If haplotype information is desired for the individual's other copy, additional clones will need to be examined. Typically, at least five clones should be examined to have more than a 90% probability of haplotyping both copies of the gene in an individual. In a particularly preferred embodiment, the nucleotide at each of polymorphic site is identified.

[0053]   In a preferred embodiment, a haplotype pair is determined for an individual by identifying the phased sequence of nucleotides at one or more of the polymorphic sites in each copy of the gene that is present in the individual. In a particularly preferred embodiment, the haplotyping method comprises identifying the phased sequence of nucleotides at each polymorphic site in each copy of the gene. When haplotyping both copies of the gene, the identifying step is preferably performed with each copy of the gene being placed in separate containers. However, it is also envisioned that if the two copies are labeled with different tags, or are otherwise separately distinguishable or identifiable, it could be possible in some cases to perform the method in the same container. For example, if first and second copies of the gene are labeled with different first and second fluorescent dyes, respectively, and an allele-specific oligonucleotide labeled with yet a third different fluorescent dye is used to assay the polymorphic site(s), then detecting a combination of the first and third dyes would identify the polymorphism in the first gene copy while detecting a combination of the second and third dyes would identify the polymorphism in the second gene copy.

[0054]   In both the genotyping and haplotyping methods, the identity of a nucleotide (or nucleotide pair) at a polymorphic site(s) may be determined by amplifying a target region(s) containing the polymorphic site(s) directly from one or both copies of the gene, or fragment thereof, and the sequence of the amplified region(s) determined by conventional methods. It will be readily appreciated by the skilled artisan that only one nucleotide will be detected at a polymorphic site in individuals who are homozygous at that site, while two different nucleotides will be detected if the individual is heterozygous for that site. The polymorphism may be identified directly, known as positive-type identification, or by inference, referred to as negative-type identification. For example, where a SNP is known

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to be guanine and cytosine in a reference population, a site may be positively determined to be either guanine or cytosine for ail individual homozygous at that site, or both guanine and cytosine, if the individual is heterozygous at that site. Alternatively, the site may be negatively determined to be not guanine (and thus cytosine/cytosine) or not cytosine (and thus guanine/guanine).

[0055]   In addition, the identity of the allele(s) present at any of the novel polymorphic sites described herein may be indirectly determined by genotyping a polymorphic site not disclosed herein that is in linkage disequilibrium with the polymorphic site that is of interest. Two sites are said to be in linkage disequilibrium if the presence of a particular variant at one site enhances the predictability of another variant at the second site (see, Stevens JC, Mol Diag 4:309-317 (1999)). Polymorphic sites in linkage disequilibrium with the presently disclosed polymorphic sites may be located in regions of the gene or in other genomic regions not examined herein. Genotyping of a polymorphic site in linkage disequilibrium with the novel polymorphic sites described herein may be performed by, but is not limited to, any of the above-mentioned methods for detecting the identity of the allele at a polymorphic site.

[0056]   The target region(s) may be amplified using any oligonucleotide-directed amplification method, including but not limited to polymerase chain reaction (PCR) (U.S. Pat. No. 4,965,188), ligase chain reaction (LCR) (Barany et al., Proc Natl Acad Sci USA 88:189-193 (1991); PCT patent application WO 90/01069), and oligonucleotide ligation assay (OLA) (Landegren et al., Science 241:1077-1080 (1988)). Oligonucleotides useful as primers or probes in such methods should specifically hybridize to a region of the nucleic acid that contains or is adjacent to the polymorphic site. Typically, the oligonucleotides are between 10 and 35 nucleotides in length and preferably, between 15 and 30 nucleotides in length. Most preferably, the oligonucleotides are 20 to 25 nucleotides long. The exact length of the oligonucleotide will depend on many factors that are routinely considered and practiced by the skilled artisan.

[0057]   Other known nucleic acid amplification procedures may be used to amplify the target region including transcription-based amplification systems (U.S. Pat. No. 5,130,238; EP 329,822; U.S. Pat. No. 5,169,766, WO 89/06700) and isothermal methods (Walker et al., Proc Natl Acad Sci USA 89:392-396 (1992)).

[0058]   A polymorphism in the target region may also be assayed before or after amplification using one of several hybridization-based methods known in the art. Typically,

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allele-specific oligonucleotides are utilized in performing such methods. The allele-specific oligonucleotides may be used as differently labeled probe pairs, with one member of the pair showing a perfect match to one variant of a target sequence and the other member showing a perfect match to a different variant. In some embodiments, more than one polymorphic site may be detected at once using a set of allele-specific oligonucleotides or oligonucleotide pairs. Preferably, the members of the set have melting temperatures within 5°C and more preferably within 2°C, of each other when hybridizing to each of the polymorphic sites being detected.

[0059]   Hybridization of an allele-specific oligonucleotide to a target polynucleotide may be performed with both entities in solution or such hybridization may be performed when either the oligonucleotide or the target polynucleotide is covalently or noncovalently affixed to a solid support. Attachment may be mediated, for example, by antibody-antigen interactions, poly-L-Lys, streptavidin or avidin-biotin, salt bridges, hydrophobic interactions, chemical linkages, UV cross-linking baking, etc. Allele-specific oligonucleotides may be synthesized directly on the solid support or attached to the solid support subsequent to synthesis. Solid-supports suitable for use in detection methods of the invention include substrates made of silicon, glass, plastic, paper and the like, which may be formed, for example, into wells (as in 96-well plates), slides, sheets, membranes, fibers, chips, dishes, and beads. The solid support may be treated, coated or derivatized to facilitate the immobilization of the allele-specific oligonucleotide or target nucleic acid.

[0060]   The genotype or haplotype for the gene of an individual may also be determined by hybridization of a nucleic sample containing one or both copies of the gene to nucleic acid arrays and subarrays such as described in WO 95/11995. The arrays would contain a battery of allele-specific oligonucleotides representing each of the polymorphic sites to be included in the genotype or haplotype.

[0061]   The identity of polymorphisms may also be determined using a mismatch detection technique, including but not limited to the RNase protection method using riboprobes (Winter *et al., Proc Natl Acad Sci USA* 82:7575 (1985); Meyers *et al., Science* 230:1242 (1985)) and proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein (Modrich P. *Ann Rev Genet* 25:229-253 (1991)). Alternatively, variant alleles can be identified by single strand conformation polymorphism (SSCP) analysis (Orita *et al., Genomics* 5:874-879 (1989); Humphries *et al.,* in *Molecular Diagnosis of Genetic Diseases,* R. Elles, ed., pp. 321-340 (1996)) or denaturing gradient gel electrophoresis

(DGGE) (Wartell *et al., Nucl Acids Res* 18:2699-2706 (1990); Sheffield *et al., Proc Natl Acad Sci USA* 86:232-236 (1989)).

[0062]   A polymerase-mediated primer extension method may also be used to identify the polymorphism(s). Several such methods have been described in the patent and scientific literature and include the "Genetic Bit Analysis" method (WO 92/15712) and the ligase / polymerase mediated genetic bit analysis (U.S. Pat. No. 5,679,524). Related methods are disclosed in WO 91/02087, WO 90/09455, WO 95/17676, U.S. Pat. Nos. 5,302,509 and 5,945,283. Extended primers containing a polymorphism may be detected by mass spectrometry as described in U.S. Pat. No. 5,605,798. Another primer extension method is allele-specific PCR (Ruafio *et al., Nucl Acids Res* 17:8392 (1989); Ruafio *et al., Nucl Acids Res* 19:6877-6882 (1991); WO 93/22456; Turki *et al., J Clin Invest* 95:1635-1641 (1995)). In addition, multiple polymorphic sites may be investigated by simultaneously amplifying multiple regions of the nucleic acid using sets of allele-specific primers as described in WO 89/10414.

[0063]   In a preferred embodiment, the haplotype frequency data for each ethnogeographic group is examined to determine whether it is consistent with Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium (D.L. Hartl *et al., Principles of Population Genomics, 3rd Ed.* (Sinauer Associates, Sunderland, MA, 1997) postulates that the frequency of finding the haplotype pair H_1/H_2 is equal to $P_{H-W} (H_1/H_2) = 2p(H_1) p (H_2)$ if $H_1 \neq H_2$ and $P_{H-W} (H_1/H_2) = p (H_1) p (H_2)$ if $H_1 = H_2$. A statistically significant difference between the observed and expected haplotype frequencies could be due to one or more factors including significant inbreeding in the population group, strong selective pressure on the gene, sampling bias, and/or errors in the genotyping process. If large deviations from Hardy-Weinberg equilibrium are observed in an ethnogeographic group, the number of individuals in that group can be increased to see if the deviation is due to a sampling bias. If a larger sample size does not reduce the difference between observed and expected haplotype pair frequencies, then one may wish to consider haplotyping the individual using a direct haplotyping method such as, for example, CLASPER System™ technology (U.S. Pat. No. 5,866,404), SMD, or allele-specific long-range PCR (Michalotos-Beloin *et al., Nucl Acids Res* 24:4841-4843 (1996)).

[0064]   In one embodiment of this method for predicting a haplotype pair, the assigning step involves performing the following analysis. First, each of the possible haplotype pairs is compared to the haplotype pairs in the reference population. Generally, only one of the

haplotype pairs in the reference population matches a possible haplotype pair and that pair
is assigned to the individual. Occasionally, only one haplotype represented in the reference
haplotype pairs is consistent with a possible haplotype pair for an individual, and in such
cases the individual is assigned a haplotype pair containing this known haplotype and a new
haplotype derived by subtracting the known haplotype from the possible haplotype pair. In
rare cases, either no haplotypes in the reference population are consistent with the possible
haplotype pairs, or alternatively, multiple reference haplotype pairs are consistent with the
possible haplotype pairs. In such cases, the individual is preferably haplotyped using a
direct molecular haplotyping method such as, for example, CLASPER System™
technology (U.S. Pat. No. 5,866,404), SMD, or allele-specific long-range PCR
(Michalotos-Beloin *et al.*, *Nucl Acids Res* 24:4841-4843 (1996)).

[0065]   The invention also provides a method for determining the frequency of a
genotype or haplotype in a population. The method comprises determining the genotype or
the haplotype pair for the gene that is present in each member of the population, wherein
the genotype or haplotype comprises the nucleotide pair or nucleotide detected at one or
more of the polymorphic sites in the gene, and calculating the frequency any particular
genotype or haplotype is found in the population. The population may be a reference
population, a family population, a same sex population, a population group, a trait
population (*e.g.*, a group of individuals exhibiting a trait of interest such as a medical
condition or response to a therapeutic treatment).

[0066]   In another aspect of the invention, frequency data for genotypes and/or
haplotypes found in a reference population are used in a method for identifying an
association between a trait and a genotype or a haplotype. The trait may be any detectable
phenotype, including but not limited to susceptibility to a disease or response to a
treatment. The method involves obtaining data on the frequency of the genotype(s) or
haplotype(s) of interest in a reference population as well as in a population exhibiting the
trait. Frequency data for one or both of the reference and trait populations may be obtained
by genotyping or haplotyping each individual in the populations using one of the methods
described above. The haplotypes for the trait population may be determined directly or,
alternatively, by the predictive genotype to haplotype approach described above.

[0067]   In another embodiment, the frequency data for the reference and/or trait
populations is obtained by accessing previously determined frequency data, which may be
in written or electronic form. For example, the frequency data may be present in a database

that is accessible by a computer. Once the frequency data is obtained, the frequencies of the genotype(s) or haplotype(s) of interest in the reference and trait populations are compared. In a preferred embodiment, the frequencies of all genotypes and/or haplotypes observed in the populations are compared. If a particular genotype or haplotype for the gene is more frequent in the trait population than in the reference population at a statistically significant amount, then the trait is predicted to be associated with that genotype or haplotype.

[0068]   In a preferred embodiment statistical analysis is performed by the use of standard ANOVA tests with a Bonferoni correction and/or a bootstrapping method that simulates the genotype phenotype correlation many times and calculates a significance value. When many polymorphisms are being analyzed a correction to factor may be performed to correct for a significant association that might be found by chance. For statistical methods for use in the methods of this invention, see: *Statistical Methods in Biology, 3rd edition*, Bailey NTJ, (Cambridge Univ. Press, 1997); *Introduction to Computational Biology*, Waterman MS (CRC Press, 2000) and *Bioinformatics*, Baxevanis AD & Ouellette BFF editors (John Wiley & Sons, Inc., 2001).

[0069]   In a preferred embodiment of the method, the trait of interest is a clinical response exhibited by a patient to some therapeutic treatment, for example, response to a drug targeting or response to a therapeutic treatment for a medical condition.

[0070]   In another embodiment of the invention, a detectable genotype or haplotype that is in linkage disequilibrium with the genotype or haplotype of interest may be used as a surrogate marker. A genotype that is in linkage disequilibrium with a genotype may be discovered by determining if a particular genotype or haplotype for the gene is more frequent in the population that also demonstrates the potential surrogate marker genotype than in the reference population at a statistically significant amount, then the marker genotype is predicted to be associated with that genotype or haplotype and then can be used as a surrogate marker in place of the genotype.

[0071]   *Definitions*. As used herein, "medical condition" includes but is not limited to any condition or disease manifested as one or more physical and/or psychological symptoms for which treatment is desirable, and includes previously and newly identified diseases and other disorders.

[0072]   As used herein, the term "clinical response" means any or all of the following: a quantitative measure of the response, no response, and adverse response (i.e., side effects).

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[0073]   In order to deduce a correlation between clinical response to a treatment and a genotype or haplotype, data is obtained on the clinical responses exhibited by a population of individuals who received the treatment, hereinafter the "clinical population". This clinical data may be obtained by analyzing the results of a clinical trial that has already been run and/or the clinical data may be obtained by designing and carrying out one or more new clinical trials.

[0074]   As used herein, the term "clinical trial" means any research study designed to collect clinical data on responses to a particular treatment, and includes but is not limited to phase I, phase II and phase III clinical trials. Standard methods are used to define the patient population and to enroll subjects.

[0075]   It is preferred that the individuals included in the clinical population have been graded for the existence of the medical condition of interest. This grading of potential patients could employ a standard physical exam or one or more lab tests. Alternatively, grading of patients could use haplotyping for situations where there is a strong correlation between haplotype pair and disease susceptibility or severity.

[0076]   The therapeutic treatment of interest is administered to each individual in the trial population and each individual's response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses and that the investigator will choose the number of responder groups (e.g., low, medium, high) made up by the various responses. In addition, the gene for each individual in the trial population is genotyped and/or haplotyped, which may be done before or after administering the treatment.

[0077]   After both the clinical and polymorphism data have been obtained, correlations between individual response and genotype or haplotype content are created. Correlations may be produced in several ways. In one method, individuals are grouped by their genotype or haplotype (or haplotype pair) (also referred to as a polymorphism group), and then the averages and standard deviations of clinical responses exhibited by the members of each polymorphism group are calculated.

[0078]   These results are then analyzed to determine if any observed variation in clinical response between polymorphism groups is statistically significant. Statistical analysis methods which may be used are described in L.D. Fisher & G. vanBelle, *Biostatistics: A Methodology for the Health Sciences* (Wiley-Interscience, New York,

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1993). This analysis may also include a regression calculation of which polymorphic sites in the gene give the most significant contribution to the differences in phenotype.

[0079]   A second method for finding correlations between haplotype content and clinical responses uses predictive models based on error-minimizing optimization algorithms. One of many possible optimization algorithms is a genetic algorithm (R. Judson, "Genetic Algorithms and Their Uses in Chemistry" in *Reviews in Computational Chemistry*, Vol. 10, pp. 1- 73, K.B. Lipkowitz & D.B. Boyd, eds. (VCH Publishers, New York, 1997). Simulated annealing (Press *et al.*, "Numerical Recipes in C: The Art of Scientific Computing", Cambridge University Press (Cambridge) 1992, Ch. 10), neural networks (E. Rich and K. Knight, "Artificial Intelligence", 2nd Edition (McGraw-Hill, New York, 1991, Ch. 18), standard gradient descent methods (Press *et al., supra* Ch. 10), or other global or local optimization approaches (see discussion in Judson, supra) could also be used.

[0080]   Correlations may also be analyzed using analysis of variation (ANOVA) techniques to determine how much of the variation in the clinical data is explained by different subsets of the polymorphic sites in the gene. ANOVA is used to test hypotheses about whether a response variable is caused by or correlated with one or more traits or variables that can be measured (Fisher & vanBelle, supra, Ch. 10).

[0081]   From the analyses described above, a mathematical model may be readily constructed by the skilled artisan that predicts clinical response as a function of genotype or haplotype content.

[0082]   The identification of an association between a clinical response and a genotype or haplotype (or haplotype pair) for the gene may be the basis for designing a diagnostic method to determine those individuals who will or will not respond to the treatment, or alternatively, will respond at a lower level and thus may require more treatment, *i.e.*, a greater dose of a drug. The diagnostic method may take one of several forms: for example, a direct DNA test (*i.e.*, genotyping or haplotyping one or more of the polymorphic sites in the gene), a serological test, or a physical exam measurement. The only requirement is that there be a good correlation between the diagnostic test results and the underlying genotype or haplotype that is in turn correlated with the clinical response. In a preferred embodiment, this diagnostic method uses the predictive haplotyping method described above.

[0083]   A computer may implement any or all analytical and mathematical operations involved in practicing the methods of the present invention. In addition, the computer may

execute a program that generates views (or screens) displayed on a display device and with which the user can interact to view and analyze large amounts of information relating to the gene and its genomic variation, including chromosome location, gene structure, and gene family, gene expression data, polymorphism data, genetic sequence data, and clinical data population data (*e.g.*, data on ethnogeographic origin, clinical responses, genotypes, and haplotypes for one or more populations). The polymorphism data described herein may be stored as part of a relational database (*e.g.*, an instance of an Oracle database or a set of ASCII flat files). These polymorphism data may be stored on the computer's hard drive or may, for example, be stored on a CD-ROM or on one or more other storage devices accessible by the computer. For example, the data may be stored on one or more databases in communication with the computer via a network.

[0084]   In other embodiments, the invention provides methods, compositions, and kits for haplotyping and/or genotyping the gene in an individual. The compositions contain oligonucleotide probes and primers designed to specifically hybridize to one or more target regions containing, or that are adjacent to, a polymorphic site. The methods and compositions for establishing the genotype or haplotype of an individual at the novel polymorphic sites described herein are useful for studying the effect of the polymorphisms in the etiology of diseases affected by the expression and function of the protein, studying the efficacy of drugs targeting , predicting individual susceptibility to diseases affected by the expression and function of the protein and predicting individual responsiveness to drugs targeting the gene product.

[0085]   In yet another embodiment, the invention provides a method for identifying an association between a genotype or haplotype and a trait. In preferred embodiments, the trait is susceptibility to a disease, severity of a disease, the staging of a disease or response to a drug. Such methods have applicability in developing diagnostic tests and therapeutic treatments for all pharmacogenetic applications where there is the potential for an association between a genotype and a treatment outcome including efficacy measurements, PK measurements and side effect measurements.

[0086]   The invention also provides a computer system for storing and displaying polymorphism data determined for the gene. The computer system comprises a computer processing unit; a display; and a database containing the polymorphism data. The polymorphism data includes the polymorphisms, the genotypes and the haplotypes identified for the gene in a reference population. In a preferred embodiment, the computer

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system is capable of producing a display showing haplotypes organized according to their evolutionary relationships.

[0087]   In another aspect, the invention provides SNP probes, which are useful in classifying people according to their types of genetic variation. The SNP probes according to the invention are oligonucleotides, which can discriminate between alleles of a SNP nucleic acid in conventional allelic discrimination assays.

[0088]   As used herein, a "SNP nucleic acid" is a nucleic acid sequence, which comprises a nucleotide that is variable within an otherwise identical nucleotide sequence between individuals or groups of individuals, thus, existing as alleles. Such SNP nucleic acids are preferably from about 15 to about 500 nucleotides in length. The SNP nucleic acids may be part of a chromosome, or they may be an exact copy of a part of a chromosome, e.g., by amplification of such a part of a chromosome through PCR or through cloning. The SNP nucleic acids are referred to hereafter simply as "SNPs". The SNP probes according to the invention are oligonucleotides that are complementary to a SNP nucleic acid.

[0089]   As used herein, the term "complementary" means exactly complementary throughout the length of the oligonucleotide in the Watson and Crick sense of the word.

[0090]   In certain preferred embodiments, the oligonucleotides according to this aspect of the invention are complementary to one allele of the SNP nucleic acid, but not to any other allele of the SNP nucleic acid. Oligonucleotides according to this embodiment of the invention can discriminate between alleles of the SNP nucleic acid in various ways. For example, under stringent hybridization conditions, an oligonucleotide of appropriate length will hybridize to one allele of the SNP nucleic acid, but not to any other allele of the SNP nucleic acid. The oligonucleotide may be labeled by a radiolabel or a fluorescent label. Alternatively, an oligonucleotide of appropriate length can be used as a primer for PCR, wherein the 3' terminal nucleotide is complementary to one allele of the SNP nucleic acid, but not to any other allele. In this embodiment, the presence or absence of amplification by PCR determines the haplotype of the SNP nucleic acid.

[0091]   Genomic and cDNA fragments of the invention comprise at least one novel polymorphic site identified herein and have a length of at least 10 nucleotides and may range up to the full length of the gene. Preferably, a fragment according to the present invention is between 100 and 3000 nucleotides in length, and more preferably between 200

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and 2000 nucleotides in length, and most preferably between 500 and 1000 nucleotides in length.

[0092]   In describing the polymorphic sites identified herein reference is made to the sense strand of the gene for convenience. However, as recognized by the skilled artisan, nucleic acid molecules containing the gene may be complementary double stranded molecules and thus reference to a particular site on the sense strand refers as well to the corresponding site on the complementary antisense strand. Thus, reference may be made to the same polymorphic site on either strand and an oligonucleotide may be designed to hybridize specifically to either strand at a target region containing the polymorphic site. Thus, the invention also includes single-stranded polynucleotides that are complementary to the sense strand of the genomic variants described herein.

[0093]   In a preferred embodiment, such kit may further comprise a DNA sample collecting means.

[0094]   In particular, the genotyping primer composition may comprise at least two sets of allele specific primer pairs. Preferably, the two genotyping oligonucleotides are packaged in separate containers.

[0095]   It is to be understood that the methods of the invention described herein generally may further comprise the use of a kit according to the invention. Generally, the methods of the invention may be performed *ex-vivo*, and such *ex-vivo* methods are specifically contemplated by the present invention. Also, where a method of the invention may include steps that may be practiced on the human or animal body, methods that only comprise those steps which are not practiced on the human or animal body are specifically contemplated by the present invention.

[0096]   Effect(s) of the polymorphisms identified herein on expression of may be investigated by preparing recombinant cells and/or organisms, preferably recombinant animals, containing a polymorphic variant of the gene. As used herein, "expression" includes but is not limited to one or more of the following: transcription of the gene into precursor mRNA; splicing and other processing of the precursor mRNA to produce mature mRNA; mRNA stability; translation of the mature mRNA into protein (including codon usage and tRNA availability); and glycosylation and/or other modifications of the translation product, if required for proper expression and function.

[0097]   To prepare a recombinant cell of the invention, the desired isogene may be introduced into the cell in a vector such that the isogene remains extrachromosomal. In such

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a situation, the gene will be expressed by the cell from the extrachromosomal location. In a preferred embodiment, the isogene is introduced into a cell in such a way that it recombines with the endogenous gene present in the cell. Such recombination requires the occurrence of a double recombination event, thereby resulting in the desired gene polymorphism. Vectors for the introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector or vector construct may be used in the invention. Methods such as electroporation, particle bombardment, calcium phosphate co-precipitation and viral transduction for introducing DNA into cells are known in the art; therefore, the choice of method may lie with the competence and preference of the skilled practitioner.

[0098]   Recombinant organisms, i.e., transgenic animals, expressing a variant gene are prepared using standard procedures known in the art. Preferably, a construct comprising the variant gene is introduced into a nonhuman animal or an ancestor of the animal at an embryonic stage, i.e., the one-cell stage, or generally not later than about the eight-cell stage. Transgenic animals carrying the constructs of the invention can be made by several methods known to those having skill in the art. One method involves transfecting into the embryo a retrovirus constructed to contain one or more insulator elements, a gene or genes of interest, and other components known to those skilled in the art to provide a complete shuttle vector harboring the insulated gene(s) as a transgene, see e.g., U.S. Pat. No. 5,610,053. Another method involves directly injecting a transgene into the embryo. A third method involves the use of embryonic stem cells.

[0099]   Examples of animals, into which the isogenes may be introduced include, but are not limited to, mice, rats, other rodents, and nonhuman primates (see "The Introduction of Foreign Genes into Mice" and the cited references therein, In: *Recombinant DNA*, Eds. J .D. Watson, M. Gilman, J. Witkowski, & M. Zoller; W.H. Freeman and Company, New York, pages 254-272). Transgenic animals stably expressing a human isogene and producing human protein can be used as biological models for studying diseases related to abnormal expression and/or activity, and for screening and assaying various candidate drugs, compounds, and treatment regimens to reduce the symptoms or effects of these diseases.

[00100]   In practicing the present invention, many conventional techniques in molecular biology, microbiology and recombinant DNA are used. These techniques are well-known and are explained in, *e.g.*, *"Current Protocols in Molecular Biology"*, Vols. I-III, Ausubel,

Ed. (1997); Sambrook *et al.*, *"Molecular Cloning: A Laboratory Manual"*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989); *"DNA Cloning: A Practical Approach"*, Vols. I and II, Glover, Ed. (1985); *"Oligonucleotide Synthesis"*, Gait, Ed. (1984); *"Nucleic Acid Hybridization"*, Hames & Higgins, Eds. (1985); *"Transcription and Translation"*, Hames & Higgins, Eds. (1984); *"Animal Cell Cu*lture*"*, Freshney, Ed. (1986); *"Immobilized Cells and Enzymes"*, IRL Press (1986); Perbal, *"A Practical Guide to Molecular Cloning"*; the series, Methods in Enzymol., Academic Press, Inc. (1984); *"Gene Transfer Vectors for Mammalian Cells"*, Miller and Calos, Eds., Cold Spring Harbor Laboratory, NY (1987); and *Methods in Enzymology*, Vols. 154 and 155, Wu & Grossman, and Wu, Eds., respectively.

[00101]   The standard control levels of the gene expression product, thus determined in the different control groups, would then be compared with the measured level of an gene expression product in a given patient. This gene expression product could be the characteristic mRNA associated with that particular genotype group or the polypeptide gene expression product of that genotype group. The patient could then be classified or assigned to a particular genotype group based on how similar the measured levels were compared to the control levels for a given group.

[00102]   As one of skill in the art will understand, there will be a certain degree of uncertainty involved in making this determination. Therefore, the standard deviations of the control group levels would be used to make a probabilistic determination and the methods of this invention would be applicable over a wide range of probability based genotype group determinations. Thus, for example and not by way of limitation, in one embodiment, if the measured level of the gene expression product falls within 2.5 standard deviations of the mean of any of the control groups, then that individual may be assigned to that genotype group. In another embodiment if the measured level of the gene expression product falls within 2.0 standard deviations of the mean of any of the control groups then that individual may be assigned to that genotype group. In still another embodiment, if the measured level of the gene expression product falls within 1.5 standard deviations of the mean of any of the control groups then that individual may be assigned to that genotype group. In yet another embodiment, if the measured level of the gene expression product is 1.0 or less standard deviations of the mean of any of the control groups levels then that individual may be assigned to that genotype group.

[00103]   Thus this process will allow the determining, with various degrees of probability, which group a specific patient should be place in and such assignment to a genotype group would then determine the risk category into which the individual should be placed.

[00104]   Methods to detect and measure mRNA levels and levels of polypeptide gene expression products are well known in the art and include the use of nucleotide microarrays and polypeptide detection methods involving mass spectrometers and/or antibody detection and quantification techniques. See also, *Human Molecular Genetics*, 2nd Edition. Tom Strachan & Andrew, Read (John Wiley and Sons, Inc. Publication, NY, 1999).

[00105]   Furthermore, detection of the concentration of the polypeptide (protein) expression product of the gene in body fluids or tissues can be used to determine the presence or absence of the polymorphism, and the relative level of the polypeptide expression product can be used to determine if the polymorphism is present in a homozygous or heterozygous state and therefore the risk category of the individual.

[00106]   As used herein, "medical condition" includes, but is not limited to, any condition or disease manifested as one or more physical and/or psychological symptoms for which treatment is desirable, and includes previously and newly-identified diseases and other disorders.

[00107]   As used herein the term "polymorphism" shall mean any sequence variant present at a frequency of >1% in a population. The sequence variant may be present at a frequency significantly greater than 1% such as 5% or 10 % or more. Also, the term may be used to refer to the sequence variation observed in an individual at a polymorphic site. Polymorphisms include nucleotide substitutions, insertions, deletions and microsatellites and may, but need not, result in detectable differences in gene expression or protein function.

[00108]   As used herein, the term "clinical response" means any or all of the following: a quantitative measure of the response, no response and adverse response, *i.e.*, side effects.

[00109]   As used herein the term "allele" shall mean a particular form of a gene or DNA sequence at a specific chromosomal location (locus).

[00110]   As used herein, the term "genotype" shall mean an unphased 5' to 3' sequence of nucleotide pair(s) found at one or more polymorphic sites in a locus on a pair of homologous chromosomes in an individual. As used herein, genotype includes a full-genotype and/or a sub-genotype.

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[00111]   As used herein, the term "polynucleotide" shall mean any RNA or DNA, which may be unmodified or modified RNA or DNA. Polynucleotides include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons.

[00112]   As used herein the term "gene" shall mean a segment of DNA that contains all the information for the regulated biosynthesis of an RNA product, including promoters, exons, introns, and other untranslated regions that control expression.

[00113]   As used herein the term "polypeptide" shall mean any polypeptide comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. Polypeptide refers to both short chains, commonly referred to as peptides, glycopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. Polypeptides include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature.

[00114]   As used herein, the term "polymorphic site" shall mean a position within a locus at which at least two alternative sequences are found in a population, the most frequent of which has a frequency of no more than 99%.

[00115]   As used herein, the term "nucleotide pair" shall mean the nucleotides found at a polymorphic site on the two copies of a chromosome from an individual.

[00116]   As used herein, the term "phased" means, when applied to a sequence of nucleotide pairs for two or more polymorphic sites in a locus, the combination of nucleotides present at those polymorphic sites on a single copy of the locus is known.

[00117]   In order to deduce a correlation between clinical response to a treatment and a genotype or haplotype, it is necessary to obtain data on the clinical responses exhibited by a population of individuals who received the treatment, hereinafter the "clinical population".

This clinical data may be obtained by analyzing the results of a clinical trial that has already been run and/or the clinical data may be obtained by designing and carrying out one or more new clinical trials.

[00118]   As used herein, the term "clinical trial" means any research study designed to collect clinical data on responses to a particular treatment, and includes, but is not limited to, Phase I, II and III clinical trials. Standard methods are used to define the patient population and to enroll subjects.

[00119]   As used herein the term "locus" shall mean a location on a chromosome or DNA molecule corresponding to a gene or a physical or phenotypic feature.

[00120]   The therapeutic treatment of interest is administered to each individual in the trial population and each individual's response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses and that the investigator will choose the number of responder groups, e.g., low, medium and high, made up by the various responses. In addition, the gene for each individual in the trial population is genotyped and/or haplotyped, which may be done before or after administering the treatment.

[00121]   *The Detection of Nucleic Acids and Proteins as Markers.* In a particular embodiment, the level of mRNA corresponding to the marker can be determined both by *in situ* and by *in vitro* formats in a biological sample using methods known in the art. The term "biological sample" is intended to include tissues, cells, biological fluids and isolates thereof, isolated from a subject, as well as tissues, cells and fluids present within a subject. Many expression detection methods use isolated RNA. For *in vitro* methods, any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from cells. See, *e.g.,* Ausubel *et al.,* Ed., *Curr. Prot. Mol. Biol.,* John Wiley & Sons, NY (1987-1999). Additionally, large numbers of tissue samples can readily be processed using techniques well-known to those of skill in the art, such as, e.g., the single-step RNA isolation process of U.S. Pat. No. 4,843,155.

[00122]   The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, PCR analyses and probe arrays. One preferred diagnostic method for the detection of mRNA levels involve contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, e.g., a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100,

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250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to a mRNA or genomic DNA encoding a marker of the present invention. Other suitable probes for use in the diagnostic assays of the invention are described herein. Hybridization of an mRNA with the probe indicates that the marker in question is being expressed.

[00123]   In one format, the mRNA is immobilized on a solid surface and contacted with a probe, for example, by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the markers of the present invention.

[00124]   An alternative method for determining the level of mRNA corresponding to a marker of the present invention in a sample involves the process of nucleic acid amplification, e.g., by RT-PCR (the experimental embodiment set forth in Mullis, U.S. Pat. No. 4,683,202 (1987); ligase chain reaction, Barany (1991), *supra*; self-sustained sequence replication, Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, Vol. 87, pp. 1874-1878 (1990); transcriptional amplification system, Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, Vol. 86, pp. 1173-1177 (1989); Q-Beta Replicase, Lizardi *et al.*, *Biol. Technology*, Vol. 6, p. 1197 (1988); rolling circle replication, U.S. Pat. No. 5,854,033 (1988); or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of the nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10-30 nucleotides in length and flank a region from about 50-200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

[00125]   For *in situ* methods, mRNA does not need to be isolated form the cells prior to detection. In such methods, a cell or tissue sample is prepared/processed using known

histological methods. The sample is then immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the marker.

[00126]   As an alternative to making determinations based on the absolute expression level of the marker, determinations may be based on the normalized expression level of the marker. Expression levels are normalized by correcting the absolute expression level of a marker by comparing its expression to the expression of a gene that is not a marker, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes, such as the actin gene or epithelial cell-specific genes. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample or between samples from different sources.

[00127]   Alternatively, the expression level can be provided as a relative expression level. To determine a relative expression level of a marker, the level of expression of the marker is determined for 10 or more samples of normal versus disease biological samples, preferably 50 or more samples, prior to the determination of the expression level for the sample in question. The mean expression level of each of the genes assayed in the larger number of samples is determined and this is used as a baseline expression level for the marker. The expression level of the marker determined for the test sample (absolute level of expression) is then divided by the mean expression value obtained for that marker. This provides a relative expression level.

[00128]   Preferably, the samples used in the baseline determination will be from patients who do not have the polymorphism. The choice of the cell source is dependent on the use of the relative expression level. Using expression found in normal tissues as a mean expression score aids in validating whether the marker assayed is specific (versus normal cells). In addition, as more data is accumulated, the mean expression value can be revised, providing improved relative expression values based on accumulated data.

[00129]   *Detection of Polypeptides*. In another embodiment of the present invention, a polypeptide corresponding to a marker is detected. A preferred agent for detecting a polypeptide of the invention is an antibody capable of binding to a polypeptide corresponding to a marker of the invention, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof, *e.g.*, Fab or F(ab')$_2$ can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct-labeling of the probe or antibody by coupling, i.e., physically linking, a detectable substance to the probe or antibody, as well as

indirect-labeling of the probe or antibody by reactivity with another reagent that is directly-labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin.

[00130]   Proteins from individuals can be isolated using techniques that are well-known to those of skill in the art. The protein isolation methods employed can, e.g., be such as those described in Harlow & Lane (1988), *supra*.

[00131]   A variety of formats can be employed to determine whether a sample contains a protein that binds to a given antibody. Examples of such formats include, but are not limited to, EIA; radioimmunoasay (RIA), Western blot analysis and ELISA. A skilled artisan can readily adapt known protein/antibody detection methods for use in determining whether cells express a marker of the present invention and the relative concentration of that specific polypeptide expression product in blood or other body tissues.

[00132]   In one format, antibodies or antibody fragments, can be used in methods, such as Western blots or immunofluorescence techniques to detect the expressed proteins. In such uses, it is generally preferable to immobilize either the antibody or proteins on a solid support. Suitable solid phase supports or carriers include any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite.

[00133]   One skilled in the art will know many other suitable carriers for binding antibody or antigen, and will be able to adapt such support for use with the present invention. For example, protein isolated from patient cells can be run on a polyacrylamide gel electrophoresis and immobilized onto a solid phase support, such as nitrocellulose. The support can then be washed with suitable buffers followed by treatment with the detectably-labeled antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support can then be detected by conventional means and this measurement translated into a level or concentration of protein in blood or another body tissue.

[00134]   The invention also encompasses kits for detecting the presence of a polypeptide or nucleic acid corresponding to a marker of the invention in a biological sample, e.g., any body fluid including, but not limited to, serum, plasma, lymph, cystic fluid, urine, stool, csf, acitic fluid or blood and including biopsy samples of body tissue. For example, the kit can

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comprise a labeled compound or agent capable of detecting a polypeptide or an mRNA encoding a polypeptide corresponding to a marker of the invention in a biological sample and means for determining the amount of the polypeptide or mRNA in the sample, *e.g.*, an antibody which binds the polypeptide or an oligonucleotide probe which binds to DNA or mRNA encoding the polypeptide. Kits can also include instructions for interpreting the results obtained using the kit.

[00135]   For antibody-based kits, the kit can comprise, *e.g.*, 1) a first antibody, e.g., attached to a solid support, which binds to a polypeptide corresponding to a marker or the invention; and, optionally; and optionally 2) a second, different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable label.

[00136]   For oligonucleotide-based kits, the kit can comprise, *e.g.*, 1) an oligonucleotide, *e.g.*, a detectably-labeled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to a marker of the invention; or 2) a pair of primers useful for amplifying a nucleic acid molecule corresponding to a marker of the invention.

[00137]   The kit can also comprise, *e.g.*, a buffering agent, a preservative or a protein-stabilizing agent. The kit can further comprise components necessary for detecting the detectable-label, e.g., an enzyme or a substrate. The kit can also contain a control sample or a series of control samples, which can be assayed and compared to the test sample. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit.

[00138]   *Kits.* The kits of the invention may contain a written product on or in the kit container. The written product describes how to use the reagents contained in the kit to predict whether a patient will respond effectively to macrolactam treatment, especially pimecrolimus treatment. In several embodiments, the use of the reagents can be according to the methods of the invention.

EXAMPLE
PHARMACOGENETIC ANALYSIS OF PATIENT RESPONSE TO THERAPY IN
CLINICAL TRIAL: POTENTIAL ASSOCIATION OF PIMECROLIMUS EFFICACY
WITH POLYMORPHISMS IN THE TNF GENE CLUSTER

[00139]   *Genotyping selected candidate genes.* Forty-six patients, corresponding to 24

patients who received tacrolimus and 22 patients who received pimecrolimus, were

analyzed for possible associations between genetic markers and treatment efficacy.

[00140]   Thirty-one unique polymorphic sites in a total of 22 genes were analyzed.

TABLE 1 lists the genotype frequencies for each SNP examined in the trial population that

consented to participation in the pharmacogenetic analysis. Each SNP is identified by three

pieces of information: (1) REF_ACC, or the Genbank accession number where genomic

sequence for the gene can be found, (2) Position, which refers to the position of the

nucleotide within the REF_ACC that was interrogated, and (3) TWT SNP#, which refers to

the number assigned by Third Wave Technologies to the assay that was designed to assess

the genotype at the position defined by items 1 and 2. Location refers to the site within the

gene that harbors the polymorphism, if known.

TABLE 1
List of polymorphisms examined in the pharmacogenomic analysis

Gene Symbol	Gene Name	TWT SNP #	REF_ACC	Allele 1 Freq.	Allele 2 Freq.	Position	Location
ACE	Angiotensin I converting enzyme	103199	AF1178569	0.50(T)	0.50(C)	10514	
ACE	Angiotensin I converting enzyme	103200	AF1178569	0.56(A)	0.44(G)	14521	
CCR2	chemokine (C-C motif) receptor 2	103240	U80924 (SEQ ID NO:10)	1.0(A)	0.00(G)	4201	3' UTR
CCR2	chemokine (C-C motif) receptor 2	47987	U80924 (SEQ ID NO:10)	0.77(G)	0.23(A)	46295	Exon 1
CCR5+	chemokine (C-C motif) receptor 5	128004	U95626	1.00(T)	0.00(A)	61785	Exon 2
CCR5	chemokine (C-C motif) receptor 5	128005	U95626	0.60(A)	0.40(G)	62035	promoter
CTLA4	cytotoxic T-lymphocyte-associated protein 4	128016	M74363	0.56(A)	0.44(G)	1241	Exon 1
ICAM1*	intercellular adhesion molecule 1	128063	X59287	0.98(A)	0.02(T)	120	Exon 2
ICAM1*	intercellular adhesion molecule 1	128062	X59288	0.98(G)	0.02(T)	659	Exon 4
IFNG	Interferon gamma	229376	J00219	0.65(A)	0.35(G)	5644	promoter
IFNGR1+	Interferon gamma receptor 1	229419	U19241	1.00(G)	0.00(A)	4020	Exon 1
IFNGR2	Interferon gamma receptor 2	252011	AP000113	0.84(A)	0.16(G)	42786	Exon 2
IL10	Interleukin 10	229400	X78437	0.67(A)	0.33(G)	8210	5'UTR
IL3	Interleukin 3	251975	AF365976	0.61(C)	0.39(T)	1990	Exon 1
IL3+	Interleukin 3	229368	AF365976	1.00(T)	0.00(C)	3622	
IL4R	Interleukin 4 receptor	229406	AF421857	0.52(C)	0.48(T)	13715	Intron 5
IL5*	Interleukin 5	229372	AF353265	0.98(G)	.02(A)	2718	
IL8	Interleukin 8	229405	AF385628	0.55(C)	0.45(T)	4501	Intron 2
ITGB2+	Integrin, beta 2 (LFA1)	128081	X64075	1.00(G)	0.00(A)	64	Exon 5
LTA	Lymphotoxin alpha	229383	M55913 (SEQ ID NO:11)	0.59(C)	0.41(A)	800	Exon 3
LTB*	Lymphotoxin beta	128095	L11016 (SEQ ID NO:12)	0.91(C)	0.09(A)	5452	

TABLE 1 (Continued)
List of polymorphisms examined in the pharmacogenomic analysis

Gene Symbol	Gene Name	TWT SNP #	REF_ACC	Allele 1 Freq.	Allele 2 Freq.	Position	Location
MICA	MHC class I polypeptide-related sequence A	229390	AF336081	0.98(A)	0.02(G)	106	Exon 2
MICA	MHC class I polypeptide-related sequence A	251974	AF336081	0.78(A)	0.22(G)	1612	Exon 5
MICA	MHC class I polypeptide-related sequence A	229395	AF336081	0.76(G)	0.24(A)	658	Exon 3
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	252013	AL035682	0.49(A)	0.51(G)	58458	
PLGC1	Phospholipase C γ-1	229389	AL022394	0.69(C)	0.31(T)	64001	
TGFB1	Transforming growth factor β-1	128146	X05839	0.73(C)	0.27(T)	629	promoter
TNF	Tumor necrosis factor α	128152	M16441 (SEQ ID NO:7)	0.79(T)	0.21(C)	3064	promoter
TNF	Tumor necrosis factor α	128150	M16441 (SEQ ID NO:7)	0.85(C)	0.15(T)	3238	promoter
TNF	Tumor necrosis factor α	128148	M16441 (SEQ ID NO:7)	0.80(G)	0.20(A)	3787	promoter
TNFSF6	tumor necrosis factor receptor superfamily, member 6	128153	X81335	0.58(G)	0.42(A)	1110	promoter

+ This SNP is not polymorphic in this patient population.

* This population is not in Hardy Weinberg equilibrium for this SNP.

[00141]   The *FKBP1A* gene (SEQ ID NO:13), which codes for FK506 Binding Protein 1A (macrophilin-12), the target of pimecrolimus and tacrolimus (FK506), was not genotyped, because the gene was found to contain no SNPs. No polymorphic sites in *FKBP1A* have been reported in public databases. We sequenced the *FKBP1A* gene to look for unknown SNPs, but found none.

[00142]   To interrogate the genotype at each locus of interest, probe sets flanking the SNP were designed using publicly available databases such as OMIM, the SNP Consortium, LocusLink and dbSNP. The probe sets were synthesized by Third Wave Technologies, Inc. (TWT, Madison WI). Genotyping was performed with 60 ng of genomic

DNA using the Invader® assay developed by Third Wave Technologies according to the manufacturer's instructions. Lyamichev V *et al., Nat Biotechnol* 17: 292-6 (1999) and Ryan D, *Mol Diagn* 4: 135-44 (1999).

[00143]   *Genotyping of ASN60THR LTA.* To confirm the results of the Third Wave Technologies genotyping assay for ASN60THR *LTA* (TWT #229383), a nested polymerase chain reaction (PCR) approach was used. All oligonucleotides were purchased from Research Genetics (Huntsville, AL). First, a 481 bp fragment was amplified using primers with the following sequences: forward (5'-acaccacctgaacgtctcttc-3'; SEQ ID NO:14) reverse (5'-tctcaatccctgaggaagtgg-3'; SEQ ID NO:15). See Genbank accession number M55913 for the complete *LTA* sequence (SEQ ID NO:11). The amplicon was purified using Microcon 100 columns (Amlicon, Beverly, MA) and used as a template to amplify a 163 bp fragment using primers with the following sequences: forward (5'-tcagccaaaccttgagccctagag–3'; SEQ ID NO:16) and reverse (5'-atgtttaccaatgaggtgagcagcaggtttgcgg-3'; SEQ ID NO:17). Both PCR reactions were carried out in a Perkin Elmer 9700 thermocycler using 80 ng of genomic DNA, 60 ng of each primer, 1.0 Unit (U) of AmpliTaq polymerase (Perkin Elmer), and 200 μM dNTP (Pharmacia, Piscataway, N.J.) in a buffer consisting of 50 mM KCl, 10mM TRIS-HCl (pH 8.3), 1.5 mM MgCl and 0.01% gelatin. The PCR cycles for both amplifications consisted of the following: 94°C, 5 min; 94°C, 30 sec, 57°C, 30 sec, and 72°C, 30 sec for 35 cycles; 72°C, 10 minutes, then 4°C. The 163 bp amplicon was digested with Fau I (New England Biolabs, Beverly MA), and electrophoresed on a 4% agarose gel. A G allele produced an uncut 163 bp fragment, while an A produced 35 and 128 bp fragments. Both the 128 and 163 bp fragments were seen in heterozygote individuals. All but two of the genotypes determined by Third Wave Technologies technology could be confirmed by restriction digest.

[00144]   *Genotyping of (-1031) TNF.* To confirm the results of the genotyping assay for *TNF* -1031, a 1113 bp fragment of the *TNF* promoter was PCR amplified and sequenced for each sample. PCR primers had the following sequences: forward (5'-TGGGAGTGAGAACTTCCCAG-3'; SEQ ID NO:18) and reverse (5'-TGAGCTCATCTGGAGGAAGC-3'; SEQ ID NO:19). See Genbank accession number M16441 for the complete sequence of human *TNF*. PCR reactions were carried out using the same conditions described above. PCR products were purified through Microcon 100 (Amlicon, Beverly, MA) columns.

[00145]   Following purification of the amplicon, 5 ng per 100 bp were used for

sequencing with the ABI Prism Dye Terminator Cycle Sequencing kit as recommended

(Perkin Elmer, Foster City, CA). Sequencing reactions contained either forward

(5'-TGGGAGTGAGAACTTCCCAG-3'; SEQ ID NO:20) or reverse

(5'-CTTAAACGTCCCCTGTATTC-3'; SEQ ID NO:21) primer and were run as follows:

96°C, 5 min; 96°C, 10 sec, 50°C, 5 sec, and 60°C, 4 min for 25 cycles; then 4°C.

Sequencing reactions were purified with Centrisep spin columns (Princeton Separation,

Adelphia, NJ) and run on the ABI 373A sequencer. DNA sequences were analyzed using

ABI Prism Sequence Analysis V3.3 software.

[00146]   *Statistical analysis.* Investigator global assessment (IGA) scores were used as

the primary marker of efficacy in the pharmacogenetic studies. IGA scores at the end of the

Investigator Blinded phase of the trial (Day 43) were used as the main time-point for

comparison. Last observation carried forward imputations were only required for one

subject at day 43. In this case, there was no IGA score available after Day 29, and the score

on this day was used in place of a day 43 IGA score. IGA scores were re-coded, as was

done to assess efficacy in the trial itself. An IGA score of 0 or 1 was considered successful

treatment and IGA scores of 2-5 were counted as treatment failure. The severity of pruritus

was also used as a marker of efficacy. Pruritus scores were dichotomized into absence

(score = 0) or presence (score = 1, 2 or 3) of pruritus. The fisher's exact model was

employed to examine the genotype effect on each efficacy variable. All statistical analyses

were done using SAS version 8.2 software.

[00147]   To correct for multiple testing, the Bonferroni correction method was applied to

all results. The equation used to correct significance scores (p values) is: Bonferroni $= P \, X$

η, where $P = $ p value for association between genotype and efficacy of treatment and $\eta = $

number of polymorphic markers genotyped in the trial.

[00148]   *Demographics trial participants.* As demonstrated in TABLE 2, the trial

subjects that consented to the pharmacogenomics (PG) analysis were representative of the

patient population in the trial overall in terms of gender, age, ethnicity, and response to

treatment.

TABLE 2
Demographics of pharmacogenomic analysis participants compared to trial subjects

	Trial[+]		Pharmacogenomics Samples[*]	
	tacrolimus	pimecrolimus	tacrolimus	pimecrolimus
Age (years)	7.8	8.1	8.7	8.5
Race				
Caucasian	31 (41%)	45 (63%)	12 (48%)	17 (77%)
Black	14 (20%)	13 (18%)	7 (28%)	4 (18%)
Oriental	4 (6%)	3 (4%)	1 (4%)	0
Other	21 (30%)	10 (14%)	5 (20%)	1 (5%)
Gender				
male	31 (44%)	31 (44%)	12 (48%)	11 (50%)
female	39 (56%)	40 (56%)	14 (52%)	11 (50%)
IGA (baseline)[$]	3: 69	3: 70	3: 24	3: 21
	2: 1	2: 1		2: 1
Efficacy (IGA)[#]	27 (40%)	22 (32%)	13 (52%)	8 (36%)
Pruritus (day 1)	0: 1 (1%)	0: 2 (3%)		
	1: 13 (19%)	1: 14 (20%)	1: 6 (24%)	1: 4 (18%)
	2: 33 (47%)	2: 25 (35%)	2: 11 (44%)	2: 9 (41%)
	3: 23 (33%)	3: 30 (42%)	3: 8 (31%)	3: 9 (41%)
Pruritus (day 43)	0: 11 (16%)	0: 9 (13%)	0: 5 (20%)	0: 2 (10%)
	1: 35 (50%)	1: 35 (49%)	1: 10 (40%)	1: 14 (67%)
	2: 19 (27%)	2: 15 (21%)	2: 7 (28%)	2: 3 (14%)
	3: 3 (4%)	3: 10 (14%)	3: 3 (12%)	3: 2 (10%)

+ There were 70 subjects in the tacrolimus arm of the trial and 71 subjects in the pimecrolimus arm of the trial

* 24 subjects that took tacrolimus and 22 patients that took pimecrolimus were analyzed in the pharmacogenomic analysis.

$ IGA scores were taken at screening.

Number and (percent) of patients that experienced efficacy, defined as an IGA score of 0 or 1 recorded on day 43 of treatment (end of blinded portion of the trial).

[00149]   *Association between (–1031) TNF and macrolactam efficacy.* As stated above, the primary efficacy variable used in statistical analysis of each genetic marker was a re-coded IGA score. A total of 31 loci in 22 genes were genotyped. Of the 31 loci that were genotyped, 5 were not polymorphic in the pharmacogenomic analysis population and were dropped from the analysis. See, TABLE 1, above. Thus, the penalty for multiple testing in this analysis is 26.

[00150]   When the trial was analyzed as a whole (both arms combined), 1 of the 26 SNPs analyzed showed a statistically significant association with efficacy. Tumor necrosis factor alpha, *TNF*, is located in the gene-rich MHC cluster on 6p21.3 (*see*, FIG. 1), and many of the genes in this region play important roles in inflammatory processes. The promoter of *TNF* is highly polymorphic. A SNP located –1031 bp from the transcription

start site of the gene associated with efficacy (p=0.04, see TABLE 3, below). These data
demonstrate that patients that harbor a C allele (either CC homozygotes or CT
heterozygotes) at (-1031) *TNF* were less likely to respond to treatment (4/17; 24% success
rate) than were TT patients (17/29; 59% success rate).

TABLE 3
Association between (–1031) *TNF* and macrolactam efficacy

Response to treatment	(-1031) *TNF* Genotype			
	CC	CT	TT	Total
No Success (IGA ≥ 2)	2 (*67%*)	11 (*79%*)	12 (*41%*)	25
Success (IGA = 0 or 1)	1 (*33%*)	3 (*21%*)	17 (*59%*)	21
Total	3	14	29	46

[00151]   TABLE 3 demonstrates the number of subjects with each genotype that did or
did not respond to treatment (referred to as "success" or "no success", respectively), as
determined by IGA scores on day 43 of treatment. Each square contains the number of
individuals with the given genotype that fit the response category, followed by the *percent*.
P value for the association is 0.04 (Fisher's Exact).

[00152]   We next examined the association between (–1031) *TNF* and efficacy for
tacrolimus and pimecrolimus treatment independently. As seen in TABLE 4, those subjects
who responded to pimecrolimus can be segregated from non-responders based on (-1031)
TNF. Only subjects with a TT genotype (SEQ ID NO:1) at (-1031) *TNF* responded to
pimecrolimus treatment. None of the ten subjects with a C allele (SEQ ID. NO:2; CC or
CT) responded to pimecrolimus treatment. While response rate among CC and CT patients
was 0%, 67% of the TTs experienced remission of their atopic dermatitis after taking
pimecrolimus.

TABLE 4
Association between (–1031) *TNF* and pimecrolimus efficacy

Response to pimecrolimus	(-1031) *TNF* Genotype			
	CC	CT	TT	Total
No success (IGA ≥ 2)	1 (*100%*)	9 (*100%*)	4 (*33%*)	14
Success (IGA = 0 or 1)	0 (*0%*)	0 (*100%*)	8 (*67%*)	8
Total	1	9	12	22

[00153]   TABLE 4 demonstrates the number of pimecrolimus treated subjects with each
genotype that did or did not respond to treatment (referred to as "success" or "no success",
respectively), as determined by IGA scores on day 43 of treatment. Each square contains

the number of individuals with the given genotype that fit the response category, followed

by the *percent*. P value for the association is 0.003 (Fisher's Exact).

[00154]   However, genotype at this locus does not appear to influence tacrolimus

efficacy. See, TABLE 5, below. Indeed, of the subjects that used tacrolimus, responders

appear to be just as likely to be TT as CT or CC. Thus, the association seen between

efficacy in the trial as a whole and (-1031) *TNF* holds only for pimecrolimus and not

macrolactams in general.

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TABLE 5
Association between (-1031) *TNF* and tacrolimus efficacy

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Response to tacrolimus	(-1031) *TNF* Genotype			
	CC	CT	TT	Total
No success (IGA ≥ 2)	1 (50%)	2 (40%)	8 (47%)	11
Success (IGA = 0 or 1)	1 (50%)	3 (60%)	9 (53%)	13
Total	2	5	17	24

[00155]   TABLE 5 demonstrates the number of tacrolimus treated subjects with each

genotype that did or did not respond to treatment (referred to as "success" or "no success",

respectively), as determined by IGA scores on day 43 of treatment. Each square contains

the number of individuals with the given genotype that fit the response category, followed

by the *percent*. There was no association found between (-1031) *TNF* genotype response to

tacrolimus; the P value for the association is 1.0 (Fisher's Exact).

[00156]   *Association between ASN60THR LTA and macrolactam efficacy.* When both

arms of the trial were analyzed together, an association with efficacy was only found with

(-1031) *TNF*. Interestingly, the data for a second polymorphism suggested a trend toward

significance. A C→A polymorphism in exon 3 of *LTA* (SEQ ID NO:3), which results in the

production of threonine instead of asparagine at the 60th amino acid of the protein

(ASN60THR), was associated with efficacy in the clinical trial (p=0.07, see TABLE 6,

below). *LTA* is tandemly located with *TNF* on 6p21.3, and numerous reports suggest that

markers in *TNF* and *LTA* are in strong linkage disequilibrium. Bouma G *et al., Scand J

Immunol* 43: 456-63 (1996); Noguchi E *et al., Am J Respir Crit Care Med* 166: 43-6

(2002); .Moffatt M & Cookson W. *Hum Molec Genet* 6: 551-4 (1997); Messer G *et al., J

Exp Med* 173: 209-19 (1991). Genotypes at this locus were determined in the

pharmacogenomic analysis participants using an Invader Assay (TWT#229383) and

confirmed by restriction digest. The data demonstrate that patients harboring an A, the

wild-type allele(SEQ ID NO:3), at this locus were more likely to respond to treatment; the

response rate among subjects with an A allele (SEQ ID NO:4); AA or AC) was 57%
(17/30), while the only 25% (4/16) of those subjects with a CC genotype experienced
efficacy in the trial.

TABLE 6
Association between ASN60THR LTA and macrolactam efficacy (both arms combined)

Response to Treatment	ASN60THR *LTA* Genotype			
	AA	AC	CC	Total
No Success (IGA ≥ 2)	3 (*30%*)	10 (*50%*)	12 (*75%*)	25
Success (IGA = 0 or 1)	7 (*70%*)	10 (*50%*)	4 (*25%*)	21
Total	10	20	16	46

[00157]   TABLE 6 demonstrates the number of subjects in the clinical trial with each
genotype that did or did not respond to treatment (referred to as "success" or "no success",
respectively), as determined by IGA scores on day 43 of treatment. Each square contains
the number of individuals with the given genotype that fit the response category, followed
by the *percent*. While a significant association was not found (P value = 0.07, Fisher's
Exact), the P value approaches significance and is suggestive of a possible association.

[00158]   We next examined the association between ASN60THR *LTA* and each arm of
the clinical trial independently. As anticipated, a significant association was found for
pimecrolimus (P=0.02, see TABLE 7, below), and a dosage effect was seen with the
polymorphism. Patients with a CC genotype at this locus responded poorly to pimecrolimus
(1/8 or 11% were successfully treated). However, patients with an AC genotype responded
better (4/10 or 40% response rate), and all patients with an AA genotype responded to the
treatment (3/3).

TABLE 7
Association between ASN60THR LTA and pimecrolimus efficacy

Response to pimecrolimus	ASN60THR *LTA* Genotype			
	AA	AC	CC	Total
No success (IGA ≥ 2)	0 (*0%*)	6 (*60%*)	8 (*89%*)	13
Success (IGA = 0 or 1)	3 (*100%*)	4 (*40%*)	1 (*11%*)	7
Total	3	10	9	22

[00159]   TABLE 7 demonstrates the number of pimecrolimus treated subjects with each
genotype that did or did not respond to treatment (referred to as "success" or "no success",
respectively), as determined by IGA scores on day 43 of treatment. Each square contains
the number of individuals with the given genotype that fit the response category, followed
by the *percent*. The P value for the association is 0.02 (Fisher's Exact).

[00160]   A similar relationship was not found between ASN60THR *LTA* and tacrolimus efficacy. As seen in TABLE 8, the presence of the A allele at ASN60THR *LTA* had no influence on response to tacrolimus. As was found with (-1031) *TNF*, the association between ASN60THR *LTA* and efficacy in the clinical trial is driven by the pimecrolimus arm of the trial.

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TABLE 8
Association between ASN60THR *LTA* and tacrolimus efficacy

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Response to tacrolimus	ASN60THR *LTA* Genotype			
	AA	AC	CC	Total
No success (IGA ≥ 2)	3 *(43%)*	4 *(40%)*	4 *(57%)*	11
Success (IGA = 0 or 1)	4 *(57%)*	6 *(60%)*	3 *(42%)*	13
Total	7	10	7	24

[00161]   TABLE 8 demonstrates the number of tacrolimus treated subjects with each genotype that did or did not respond to treatment (referred to as "success" or "no success", respectively), as determined by IGA scores on day 43 of treatment. Each square contains the number of individuals with the given genotype that fit the response category, followed by the *percent*. There was no association found between ASN60THR *LTA* genotype and response to tacrolimus; the P value for the association is 1.0 (Fisher's Exact).

[00162]   *Association between polymorphic markers and relief from pruritus.* A secondary efficacy variable used in the trial was pruritus. An analysis was done to see if ASN60THR *LTA* and (-1031) *TNF* associated with this efficacy variable (assessed on day 43). Results of the analysis for ASN60THR *LTA* and (-1031) *TNF* are shown in TABLES 9 and 10, respectively.

[00163]   Few subjects (7/46) in the pharmacogenomics group experienced relief from itching. Findings for the primary efficacy variable (IGA) suggested that the presence of a C allele at ASN60THR *LTA* decreased the likelihood of response to treatment. While a statistically significant association between ASN60THR *LTA* and pruritus was not found, no subject that was homozygous CC at this locus experienced relief from itching as a result of treatment in this trial. For (-1031) *TNF*; while analysis of the primary efficacy variable IGA suggested that TT subjects were far more likely to respond to treatment, the same cannot be said when pruritus scores are analyzed for the trial as a whole. The two arms of the trial could not be separated for analysis of pruritus because the number of responders is too small. Only two pimecrolimus subjects in the pharmacogenomic analysis samples

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experienced relief from itching. However, both of the pimecrolimus subjects that experienced efficacy with respect to pruritus were TT at (-1031) *TNF*.

TABLE 9
Association between ASN60THR *LTA* and pruritus (both arms combined).

| Response to treatment | ASN60THR *LTA* Genotype | | | |
	AA	AC	CC	Total
No Success (Pru=1,2,3)	7 (70%)	16 (80%)	16 (100%)	39
Success (Pru=0)	3 (30%)	4 (20%)	0 (0%)	7
Total	10	16	16	46

[00164]   TABLE 9 demonstrates the number of subjects with each genotype at the ASN60THR *LTA* locus studied that did or did not respond to treatment, as determined by pruritus scores on day 43 of treatment. Treatment was considered successful if the patient reported no pruritus (score = 0). Each square contains the number of individuals with the given genotype that fit the response category, followed by the *percent*. A significant association was not found between ASN60THR *LTA* genotype and relief from pruritus (P=0.06, Fisher's Exact), however the P value for the association approached significance.

TABLE 10
Association between (-1031) *TNF* and pruritus following treatment
(both arms combined)

| Response to treatment | (-1031) *TNF* Genotype | | | |
	CC	CT	TT	Total
No success (Pru=1,2,3)	3 (100%)	12 (86%)	24 (83%)	39
Success (Pru = 0)	0 (0%)	2 (14%)	5 (17%)	7
Total	3	14	29	46

[00165]   TABLE 10 demonstrates the number of subjects with each genotype at (-1031) *TNF* that did or did not respond to treatment, as determined by pruritus scores on day 43 of treatment. Treatment was considered successful if the patient reported no pruritus (score = 0). Each square contains the number of individuals with the given genotype that fit the response category, followed by the *percent*. No association was found (P=1.0, Fisher's Exact).

[00166]   *Genotypes at (-1031) TNF and ASN60THR LTA*. Genotypes of anonymized patients at these two 6p21.3 loci were obtained. Only subjects with a TT genotype at (-1031) TNF responded to pimecrolimus therapy. The observation that individuals that are TT homozygotes for (-1031) TNF are either AA homozygotes, AC heterozygotes, or CC

homozygotes or for ASN60THR LTA show that (-1031) TNF and ASN60THR are not in

complete linkage disequilibrium.

TABLE 11

Nucleotide sequence surrounding the ASN60THR LTA and (-1031) TNF polymorphisms

Gene	Allele 1	Allele 2	Surrounding Sequence
LTA	C	A	GTGAGCAGCAGGTTTGAGG[C,A]TGCTGTGGG CAAGATGCATCTTGGGGTG (SEQ ID NOS: 3 and 4)
TNF	T	C	AGCAAAGGAGAAGCTGAGAAGA[T,C]GAAGG AAAAGTCAGGGTCTGGAGGGGCGGG (SEQ ID NOS: 1 and 2)

[00167]   *Associations between pimecrolimus and tacrolimus and other markers typed.*

All of the other molecular markers typed were checked to see if they segregated responders

from non-responders for each arm of the trial independently. No additional associations

were found for pimecrolimus, though one association was found for tacrolimus. A G→A

polymorphism in the coding region of *CCR2* (SEQ ID NO:6; see TWT #47987), which

results in a change in a valine to isoleucine (VAL64ILE), associated with response to

treatment with tacrolimus (p=0.04, see TABLE 11, above). The P value for the association

between pimecrolimus efficacy and VAL64ILE *CCR2* was 1.0 (Fisher's Exact).

[00168]   *CCR2* encodes chemokine (C-C) motif receptor 2, a receptor for monocyte

chemoattractant protein-1 and a chemokine that mediates monocyte infiltration in

inflammatory diseases. *CCR2* encodes for both known isoforms of the receptor for

monocyte chemoattractant protein-1 (MCP-1, also known as SCYA2), a chemokine which

specifically mediates monocyte chemotaxis. MCP-1 has been shown to be involved with

inflammatory diseases like rheumatoid arthritis and atherosclerosis. Boring L *et al., Nature*

394:894-7 (1998). The VAL64ILE polymorphism occurs in the first transmembrane region

of CCR2 and has been studied in the context of HIV-1 infection and AIDS. Mummidi S *et*

al., Nature Med 4: 786-93 (1998). The association found was p=0.04.

[00169]   These data demonstrate that an A allele at VAL64ILE *CCR2* were more likely

to respond to tacrolimus treatment; 8/10 or 80% of AG subjects experienced efficacy, as

compared to 4/13 or 31% of GG subjects. (There were no AA subjects in the population).

This association is weaker than the associations seen with pimecrolimus efficacy,

particularly for (-1031) *TNF* and pimecrolimus efficacy (p=0.003).

TABLE 12
Association between VAL63ILE *CCR2* (TWT# 47987) and tacrolimus efficacy

	VAL63ILE *CCR2* Genotype			
Response to tacrolimus	AA	AG	GG	Total
No success (IGA ≥ 2)	0	2 *(20%)*	9 *(69%)*	11
Success (IGA = 0 or 1)	0	8 *(80%)*	4 *(31%)*	12
Total	0	10	13	23

[00170] TABLE 12 demonstrates the number of tacrolimus treated subjects with each genotype at VAL63ILE *CCR2* that did or did not respond to treatment (determined by IGA scores on day 43 of treatment). Each square contains the number of individuals with the given genotype that fit the response category, followed by the *percent*. P value for the association was 0.04 (Fisher's Exact).

[00171] *LTB* (SEQ ID NO 12; see TWT # 128095) is also located in the *TNF* gene cluster on 6p21.3. A polymorphism in *LTB* was also typed in this analysis (SEQ ID NO:22). As shown below, however, there is no association between TWT#128095 and efficacy in the clinical trial. No association was found when pimecrolimus and tacrolimus subjects were analyzed independently, either.

TABLE 13
Relationship between efficacy in trial (both arms combined) and *LTB* (TWT#128095);
data demonstrate no association (p=1.0, Fisher's Exact)

	LTB (TWT#128095)		
Response to treatment	AC	CC	Total
No success (IGA ≥ 2)	3 *(50%)*	20 *(54%)*	23
Success (IGA = 0 or 1)	3 *(50%)*	17 *(46%)*	20
Total	16	26	43

TABLE 14
Relationship between efficacy in among pimecrolimus-treated subjects and *LTB*
(TWT#128095); data demonstrate no association (p=1.0, Fisher's Exact)

	LTB (TWT#128095)		
Response to pimecrolimus	AC	CC	Total
No success (IGA ≥ 2)	0 *(50%)*	12 *(63%)*	12
Success (IGA = 0 or 1)	1 *(100%)*	7 *(37%)*	8
Total	1	19	20

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TABLE 15

Relationship between efficacy in among tacrolimus-treated subjects and *LTB* (TWT#128095); data demonstrate no association (p=1.0, Fisher's Exact)

Response to tacrolimus	*LTB* (TWT#128095)		
	AC	CC	Total
No success (IGA ≥ 2)	3 (*60%*)	8 (*44%*)	11
Success (IGA = 0 or 1)	2 (*40%*)	10 (*56%*)	12
Total	5	18	23

[00172] These data help to localize the genetic region that is responsible for response to pimecrolimus; the biologically relevant polymorphism does not appear to be localized proximal to *TNF*.

[00173] In summary, either *LTA* or *TNF* plays an important role in determining efficacy of pimecrolimus in the treatment of pediatric atopic dermatitis. By contrast, response to tacrolimus appears to be influenced by other biological pathways. Thus, different biological mechanisms influence response to pimecrolimus and tacrolimus. While pimecrolimus and tacrolimus have substantial structural similarities and both target macrophilin-12 with the ultimate effect of inhibiting calcineurin, the two compounds are known to have distinct properties. Nghiem P *et al., J Am Acad Dermatol* 46: 228-241 (2002) and references cited therein.

[00174] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. In addition, all GenBank accession numbers, Unigene Cluster numbers and protein accession numbers cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each such number was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[00175]   The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The present invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

<div align="center">CLAIMS</div>

We claim:

1. The use of pimecrolimus in the manufacture of a medicament for the treatment of
    inflammatory disease in a selected patient population, wherein the patient
    population is selected on the basis of the genotype of the patients at a genetic locus
    from the TNF gene cluster indicative of efficacy of pimecrolimus in treating
    inflammatory disease.

2. The use of tacrolimus in the manufacture of a medicament for the treatment of
    inflammatory disease in a selected patient population, wherein the patient
    population is selected on the basis of the genotype of the patients at the *CCR2*
    genetic locus indicative of efficacy of tacrolimus in treating inflammatory disease.

3. A method for treating a condition in a subject, wherein the condition is selected
    from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory
    bowel disease, rheumatoid arthritis or other condition for which pimecrolimus or
    tacrolimus is indicated, comprising the steps of:

    (a)   obtaining the genotype of a subject at a genetic locus from the TNF gene
          cluster indicative of efficacy of a selected macrolactam formulation in
          treating the condition;

    (b)   administering either the selected macrolactam formulation or an alternative
          treatment for the condition to the subject.

4. The method of claim 3, wherein the selected macrolactam formulation comprises
    pimecrolimus.

5. The method of claim 3, wherein the selected macrolactam formulation comprises
    tacrolimus.

6. The method of any one of claims 3 to 5, wherein the genetic locus is the human
    TNF gene or a gene in a vertebrate species homologous to the human *TNF* gene.

7.   The use of claim 1 or the method of any one of claims 3 to 6, wherein the genetic locus is the (-1031) *TNF* locus of the human *TNF* gene or a corresponding locus in a vertebrate species homologous to the human *TNF* gene.

8.   The method of any one of claims 3, 4, 6 or 7, wherein the selected macrolactam formulation comprises pimecrolimus, when the (-1031) *TNF* genetic locus has a TT genotype.

9.   The method of any one of claims 3 to 7, wherein the alternative treatment for the condition is selected from the group consisting of a high dose of pimecrolimus, pimecrolimus and an alternative immunosuppressant, and an alternative immunosuppressant, when the (-1031) *TNF* locus has a CC or CT genotype.

10.   The method of claim 9, wherein the alternative immunosuppressant is selected from the group consisting of hydrocortisone, cyclosporine, tacrolimus and sirolimus.

11.   The use of claim 1 or the method of claim 3, wherein the genetic locus is the human *LTA* gene or a gene in a vertebrate species homologous to the human *LTA* gene.

12.   The use of claim 1 or 11 or the method of claim 3 or 11, wherein the genetic locus is the ASN60THR *LTA* locus of the human *LTA* gene or a corresponding locus in a vertebrate species homologous to the human *LTA* gene.

13.   The method of any one of claims 3, 11 or 12, wherein the selected macrolactam formulation comprises pimecrolimus, when the ASN60THR *LTA* genetic locus has an AA or AC genotype.

14.   The method of any one of claims 3, 11 or 12, wherein the alternative treatment for the condition is selected from the group consisting of a high dose of pimecrolimus, pimecrolimus and an alternative immunosuppressant, and an alternative immunosuppressant, when the ASN60THR *LTA* locus has a CC genotype.

15.   The method of claim 14, wherein the alternative immunosuppressant is selected from the group consisting of hydrocortisone, cyclosporine, tacrolimus and sirolimus.

16.   A method for treating a condition in a subject, wherein the condition is selected from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for which pimecrolimus or tacrolimus is indicated, comprising the steps of:

      (a)   obtaining the genotype of a subject at a genetic locus indicative of efficacy of a selected macrolactam formulation in treating the condition, where the genetic locus is the human *CCR2* gene or a gene in a vertebrate species homologous to the human *CCR2* gene;

      (b)   administering either the selected macrolactam formulation or an alternative treatment for the condition to the subject.

17.   The use of claim 2 or the method of claim 16, wherein the genetic locus is the VAL64ILE *CCR2* locus of the human *CCR2* gene or a corresponding locus in a vertebrate species homologous to the human *CCR2* gene.

18.   The method of claim 16 or 17, wherein the selected macrolactam formulation comprises tacrolimus, when the VAL64ILE *CCR2* genetic locus has a GG genotype.

19.   The method of claim 16 or 17, wherein the alternative treatment for the condition is selected from the group consisting of a high dose of tacrolimus, tacrolimus and an alternative immunosuppressant, and an alternative immunosuppressant, when the VAL64ILE *CCR2* locus has an AG genotype.

20.   The method of claim 16, 17 or 19, wherein the alternative immunosuppressant is selected from the group consisting of hydrocortisone, cyclosporine, pimecrolimus and sirolimus.

21.   A method for treating a condition in a subject, wherein the condition is selected
      from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory
      bowel disease, rheumatoid arthritis or other condition for which pimecrolimus is
      indicated, comprising the steps of:

      (a)   obtaining a measurement of the level of TNF-α mRNA in a sample from a
            subject, where the sample is taken from non-inflamed tissue; and

      (b)   either:

            (i)   administering a pimecrolimus formulation to the subject when the
                  level of TNF-α mRNA in the sample is low or normal; or

            (ii) administering either (A) a pimecrolimus formulation in combination
                  with another immunosuppressant or (B) another immunosuppressant
                  to the subject when the level of TNF-α mRNA in the sample is
                  elevated.

22.   A method for treating a condition in a subject, wherein the condition is selected
      from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory
      bowel disease, rheumatoid arthritis or other condition for which pimecrolimus is
      indicated, comprising the steps of:

      (a)   obtaining a measurement of the level of TNF-α protein in a sample from a
            subject, where the sample is taken from non-inflamed tissue; and

      (b)   either:

            (i)   administering a pimecrolimus formulation to the subject when the
                  level of TNF-α protein in the sample is low or normal; or

            (ii) administering either (A) a pimecrolimus formulation in combination
                  with another immunosuppressant or (B) another immunosuppressant
                  to the subject when the level of TNF-α protein in the sample is
                  elevated.

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23.   A method for determining a treatment strategy for a condition in a subject, wherein
      the condition is selected from the group consisting of atopic dermatitis, psoriasis,
      asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for
      which pimecrolimus is indicated, said method comprises analyzing the level of
      TNF-α mRNA or TNF-α protein in a sample from the subject; and wherein the
      treatment strategy comprises the selection of a pimecrolimus formulation as
      treatment when the level of TNF-α mRNA or TNF-α protein in the sample is low or
      normal; or the selection of either (A) a pimecrolimus formulation in combination
      with another immunosuppressant or (B) another immunosuppressant as treatment
      when the level of TNF-α mRNA or TNF-α protein in the sample is elevated.

24.   A method for choosing subjects for inclusion in a clinical trial for determining the
      efficacy of a pimecrolimus formulation, comprising the steps of:

      (a)   interrogating the genotype of a subject at the (-1031) TNF polymorphism
            locus;

      (b)   then:

            (i)   including in the trial if they are TT at this locus;

            (ii)   excluded if they are CC or CT at this locus; or

            (iii) both (i) and (ii).

25.   A kit for use in determining a treatment strategy for a condition, wherein the
      condition is selected from the group consisting of atopic dermatitis, psoriasis,
      asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for
      which pimecrolimus or tacrolimus is indicated, comprising:

      (a)   a reagent for detecting a biomarker of efficacy of treatment of the condition
            by a macrolactam formulation;

      (b)   a container for the reagent; and

      (c)   a written product on or in the container describing the use of the biomarker
            in determining a treatment strategy for the condition.

26.   The kit of claim 25, wherein the biomarker is a genetic polymorphism in a gene
      selected from the group consisting of *TNF*, *LTA* and *CCR2*.

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27.   The kit of claim 25 or 26, wherein the reagent for detecting the biomarker is a set of primer pairs that hybridize to a polynucleotide on either the side of the genetic polymorphism in the gene selected from the group consisting of *TNF*, *LTA* and *CCR2* and which define a nucleotide region that spans the genetic polymorphism.

28.   The kit of claim 25, wherein the biomarker is the level of TNF-α mRNA in a sample from a subject to be treated.

29.   The kit of claim 25, wherein the biomarker is the level of TNF-α protein in a sample from a subject to be treated.

30.   A method for determining a treatment strategy for a condition in a subject, wherein the condition is selected from the group consisting of atopic dermatitis, psoriasis, asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for which pimecrolimus or tacrolimus is indicated, said method comprises analyzing the genotype of a subject at a genetic locus from the TNF gene cluster indicative of efficacy of a selected macrolactam formulation in treating the condition.

31.   The method of claim 30, wherein the genetic locus is the human *TNF* gene or a gene in a vertebrate species homologous to the human *TNF* gene.

32.   The method of claim 30 or 31, wherein the genetic locus is the (-1031) *TNF* locus of the human *TNF* gene or a corresponding locus in a vertebrate species homologous to the human *TNF* gene.

33.   The method of any one of claims 30 to 32, wherein the treatment strategy comprises the selection of a macrolactam formulation comprising pimecrolimus as treatment, when the (-1031) *TNF* genetic locus has a TT genotype.

34.   The method of any one of claims 30 to 32, wherein the treatment strategy comprises the selection of a high dose of pimecrolimus, pimecrolimus and an alternative immunosuppressant, or an alternative immunosuppressant as treatment, when the (-1031) *TNF* locus has a CC or CT genotype.

35.   The method of claim 34, wherein the alternative immunosuppressant is selected
        from the group consisting of hydrocortisone, cyclosporine, tacrolimus and
        sirolimus.

36.   The method of claim 30, wherein the genetic locus is the human *LTA* gene or a gene
        in a vertebrate species homologous to the human *LTA* gene.

37.   The method of claim 36, wherein the genetic locus is the ASN60THR *LTA* locus of
        the human *LTA* gene or a corresponding locus in a vertebrate species homologous to
        the human *LTA* gene.

38.   The method of claim 36 or 37, wherein the treatment strategy comprises the
        selection of a macrolactam formulation comprising pimecrolimus as treatment,
        when the ASN60THR *LTA* genetic locus has an AA or AC genotype.

39.   The method of claim 36 or 37, wherein the treatment strategy comprises the
        selection of a high dose of pimecrolimus, pimecrolimus and an alternative
        immunosuppressant, or an alternative immunosuppressant as treatment, when the
        ASN60THR *LTA* locus has a CC genotype.

40.   The method of claim 39, wherein the alternative immunosuppressant is selected
        from the group consisting of hydrocortisone, cyclosporine, tacrolimus and
        sirolimus.

41.   A method for determining a treatment strategy for a condition in a subject, wherein
      the condition is selected from the group consisting of atopic dermatitis, psoriasis,
      asthma, inflammatory bowel disease, rheumatoid arthritis or other condition for
      which pimecrolimus or tacrolimus is indicated, said method comprises analyzing the
      genotype of a subject at a genetic locus indicative of efficacy of a selected
      macrolactam formulation in treating the condition, where the genetic locus is the
      human *CCR2* gene or a gene in a vertebrate species homologous to the human
      CCR2 gene.

42.   The method of claim 41, wherein the genetic locus is the VAL64ILE *CCR2* locus of
      the human *CCR2* gene or a corresponding locus in a vertebrate species homologous
      to the human *CCR2* gene.

43.   The method of claim 41 or 42, wherein the treatment strategy comprises the
      selection of a macrolactam formulation comprising tacrolimus as treatment, when
      the VAL64ILE *CCR2* genetic locus has a GG genotype.

44.   The method of claim 41 or 42, comprising the selection of a high dose of
      tacrolimus, tacrolimus and an alternative immunosuppressant, or an alternative
      immunosuppressant as treatment, when the VAL64ILE *CCR2* locus has an AG
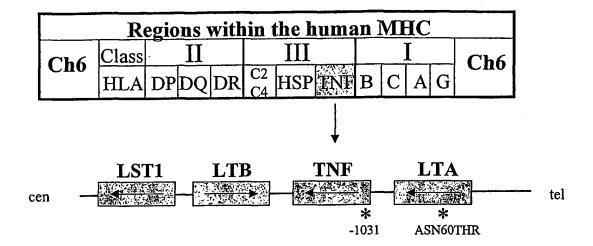   genotype.

45.   The method of claim 44, wherein the alternative immunosuppressant is selected
      from the group consisting of hydrocortisone, cyclosporine, pimecrolimus and
      sirolimus.

FIG. 1

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      Ide, Susan
      Lavedan, Christian

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atattgttcc ctattttgcc agtgggaact cctaaatcaa attggcttct aatcaaagct 1861
tttaaaccct attggtaaag aatggaaggt ggagaagctc cctgaagtaa gcaaagactt 1921
tcctcttagt cgagccaagt taagaatgtt cttatgttgc ccagtgtgtt tctgatctga 1981
tgcaagcaag aaacactggg cttctagaac caggcaactt gggaactaga ctcccaagct 2041
ggactatggc tctactttca ggccacatgg ctaaagaagg tttcagaaag aagtgggggac 2101
agagcagaac tttcaccttc atatatttgt atgatcctaa tgaatgcata aaatgttaag 2161
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 1               5                  10                  15

cta cac ctc ctc ctt ctg ggg ctg ctg ctg gtt ctg ctg cct ggg gcc     96
Leu His Leu Leu Leu Leu Gly Leu Leu Leu Val Leu Leu Pro Gly Ala
            20                  25                  30

cag ggg ctc cct ggt gtt ggc ctc aca cct tca gct gcc cag act gcc    144
Gln Gly Leu Pro Gly Val Gly Leu Thr Pro Ser Ala Ala Gln Thr Ala
        35                  40                  45

cgt cag cac ccc aag atg cat ctt gcc cac agc acc ctc aaa cct gct    192
Arg Gln His Pro Lys Met His Leu Ala His Ser Thr Leu Lys Pro Ala
    50                  55                  60

gct cac ctc att gga gac ccc agc aag cag aac tca ctg ctc tgg aga    240
Ala His Leu Ile Gly Asp Pro Ser Lys Gln Asn Ser Leu Leu Trp Arg
 65                  70                  75                  80

gca aac acg gac cgt gcc ttc ctc cag gat ggt ttc tcc ttg agc aac    288
Ala Asn Thr Asp Arg Ala Phe Leu Gln Asp Gly Phe Ser Leu Ser Asn
                85                  90                  95

aat tct ctc ctg gtc ccc acc agt ggc atc tac ttc gtc tac tcc cag    336
Asn Ser Leu Leu Val Pro Thr Ser Gly Ile Tyr Phe Val Tyr Ser Gln
            100                 105                 110

gtg gtc ttc tct ggg aaa gcc tac tct ccc aag gcc acc tcc tcc cca    384
Val Val Phe Ser Gly Lys Ala Tyr Ser Pro Lys Ala Thr Ser Ser Pro
            115                 120                 125

ctc tac ctg gcc cat gag gtc cag ctc ttc tcc tcc cag tac ccc ttc    432
Leu Tyr Leu Ala His Glu Val Gln Leu Phe Ser Ser Gln Tyr Pro Phe
        130                 135                 140

cat gtg cct ctc ctc agc tcc cag aag atg gtg tat cca ggg ctg cag    480
His Val Pro Leu Leu Ser Ser Gln Lys Met Val Tyr Pro Gly Leu Gln
145                 150                 155                 160

gaa ccc tgg ctg cac tcg atg tac cac ggg gct gcg ttc cag ctc acc    528
Glu Pro Trp Leu His Ser Met Tyr His Gly Ala Ala Phe Gln Leu Thr
                165                 170                 175

cag gga gac cag cta tcc acc cac aca gat ggc atc ccc cac cta gtc    576
Gln Gly Asp Gln Leu Ser Thr His Thr Asp Gly Ile Pro His Leu Val
            180                 185                 190

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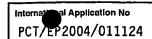<223> Lymphotoxin beta (LTB) mRNA coding region

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ggg agg ggt tcc ctc ctg cta gct gtg gca gga gcc act tct ctg gtg   98
Gly Arg Gly Ser Leu Leu Leu Ala Val Ala Gly Ala Thr Ser Leu Val
 15              20                  25                  30

acc ttg ttg ctg gcg gtg cct atc act gtc ctg gct gtg ctg gcc tta  146
Thr Leu Leu Leu Ala Val Pro Ile Thr Val Leu Ala Val Leu Ala Leu
                 35                  40                  45

gtg ccc cag gat cag gga gga ctg gta acg gag acg gcc gac ccc ggg  194
Val Pro Gln Asp Gln Gly Gly Leu Val Thr Glu Thr Ala Asp Pro Gly
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gca cag gcc cag caa gga ctg ggg ttt cag aag ctg cca gag gag gag  242
Ala Gln Ala Gln Gln Gly Leu Gly Phe Gln Lys Leu Pro Glu Glu Glu
         65                  70                  75

cca gaa aca gat ctc agc ccc ggg ctc cca gct gcc cac ctc ata ggc  290
Pro Glu Thr Asp Leu Ser Pro Gly Leu Pro Ala Ala His Leu Ile Gly
     80                  85                  90

gct ccg ctg aag ggg cag ggg cta ggc tgg gag acg acg aag gaa cag  338
Ala Pro Leu Lys Gly Gln Gly Leu Gly Trp Glu Thr Thr Lys Glu Gln
 95              100                 105                 110

gcg ttt ctg acg agc ggg acg cag ttc tcg gac gcc gag ggg ctg gcg  386
Ala Phe Leu Thr Ser Gly Thr Gln Phe Ser Asp Ala Glu Gly Leu Ala
                 115                 120                 125

ctc ccg cag gac ggc ctc tat tac ctc tac tgt ctc gtc ggc tac cgg  434
Leu Pro Gln Asp Gly Leu Tyr Tyr Leu Tyr Cys Leu Val Gly Tyr Arg
             130                 135                 140

ggc cgg gcg ccc cct ggc ggc ggg gac ccc cag ggc cgc tcg gtc acg  482
Gly Arg Ala Pro Pro Gly Gly Gly Asp Pro Gln Gly Arg Ser Val Thr
             145             150                 155

ctg cgc agc tct ctg tac cgg gcg ggg ggc gcc tac ggg ccg ggc act  530
Leu Arg Ser Ser Leu Tyr Arg Ala Gly Gly Ala Tyr Gly Pro Gly Thr
 160                 165                 170

ccc gag ctg ctg ctc gag ggc gcc gag acg gtg act cca gtg ctg gac  578
Pro Glu Leu Leu Leu Glu Gly Ala Glu Thr Val Thr Pro Val Leu Asp
175                 180                 185                 190

ccg gcc agg aga caa ggg tac ggg cct ctc tgg tac acg agc gtg ggg  626
Pro Ala Arg Arg Gln Gly Tyr Gly Pro Leu Trp Tyr Thr Ser Val Gly
                 195                 200                 205

ttc ggc ggc ctg gtg cag ctc cgg agg ggc gag agg gtg tac gtc aac  674
Phe Gly Gly Leu Val Gln Leu Arg Arg Gly Glu Arg Val Tyr Val Asn
             210                 215                 220

atc agt cac ccc gat atg gtg gac ttc gcg aga ggg aag acc ttc ttt  722
Ile Ser His Pro Asp Met Val Asp Phe Ala Arg Gly Lys Thr Phe Phe
         225                 230                 235

ggg gcc gtg atg gtg ggg tga gggaatatga gtgcgtggtg cgagtgcgtg   773
Gly Ala Val Met Val Gly  *
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 1   5   10   15

ccc aag cgc ggc cag acc tgc gtg gtg cac tac acc ggg atg ctt gaa   96
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
            20   25   30

gat gga aag aaa ttt gat tcc tcc cgg gac aga aac aag ccc ttt aag 144
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
        35   40   45

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Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
    50   55   60

gcc cag atg agt gtg ggt cag aga gcc aaa ctg act ata tct cca gat 240
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
 65   70   75   80

tat gcc tat ggt gcc act ggg cac cca ggc atc atc cca cca cat gcc 288
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
                85   90   95

act ctc gtc ttc gat gtg gag ctt cta aaa ctg gaa tga   327
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<400> 21
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBL, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 288 088 A (TEIJIN LIMITED) 26 October 1988 (1988-10-26) claims 19,20	25-29
X	US 6 235 723 B1 (DEAN NICHOLAS M) 22 May 2001 (2001-05-22) column 29, lines 32-34 column 31, lines 5-9	25-29
A	HIGUCHI T ET AL: "POLYMORPHISM OF THE 5'-FLANKING REGION OF THE HUMAN TUMOR NECROSIS FACTOR (TNF)-ALPHA GENE IN JAPANESE" TISSUE ANTIGENS, MUNKSGAARD, COPENHAGEN, DK, vol. 51, no. 6, 1998, pages 605-612, XP008020598 ISSN: 0001-2815	

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 2005	10/03/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Leber, T

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH ET AL: "Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 277, 15 August 1997 (1997-08-15), pages 959-965, XP002099015 ISSN: 0036-8075	
A	HOLWEG C T J ET AL: "Clinical impact of cytokine gene polymorphisms in heart and lung transplantation" JOURNAL OF HEART AND LUNG TRANSPLANTATION, MOSBY-YEAR BOOK, INC., ST LOUIS, MO, US, vol. 23, no. 9, September 2004 (2004-09), pages 1017-1026, XP004595231 ISSN: 1053-2498	
A	ABRAHAM L J ET AL: "HAPLOTYPIC POLYMORPHISMS OF THE TNFB GENE" IMMUNOGENETICS, vol. 33, no. 1, 1991, pages 50-53, XP009041878 ISSN: 0093-7711	
A	LEE BENHUR ET AL: "Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4" JOURNAL OF VIROLOGY, vol. 72, no. 9, September 1998 (1998-09), pages 7450-7458, XP002312893 ISSN: 0022-538X	
A	WO 02/089796 A (NOVARTIS AG; CHIBOUT, SALAH-DINE; CORDIER, ANDRE; KEHREN, JEANNE; MOOR) 14 November 2002 (2002-11-14)	

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			EP	1126025 A2	22-08-2001
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